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(54) Title: VACCINE COMPRISING MIXED preS1 + preS2 + S AND CORE PARTICLE

(57) Abstract

To produce hepatitis B virus (HBV) surface and core proteins as mixed particles, DNA encoding HBV proteins is expressed in a single recombinant yeast. To form particles with substantially reduced carbohydrate, DNA encoding HBV proteins is expressed in a single recombinant yeast which is glycosylation deficient. These HBV proteins display, on the same mixed particle, antigenic sites genetically encoded by the envelope domains (including preS and S) and the core antigen. The particles also contain substantially reduced nucleic acid content. These particles are useful as a vaccine for active and passive treatment or prevention of HBV disease and/or infection and serologically related agents including surface protein antigenic variants (especially in populations hypo- or non-responsive to other HBV vaccines), and also as reagents for use in diagnostic tests.

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TITLE OF THE INVENTION

VACCINE COMPRISING MIXED preS1+preS2+S AND CORE PARTICLE

BACKGROUND OF THE INVENTION

Hepatitis B virus (HBV) is the infectious agent responsible for several varieties of human liver disease. Many individuals who are infected by HBV suffer through an acute phase of disease, which is followed by recovery. However, a percentage of infected individuals fail to clear their infection, thereby becoming chronic carriers of the infection. HBV infection is endemic in many parts of the world, with a high incidence of infection occurring

perinatally from chronically infected mothers to their newborns who themselves often remain chronically infected. The number worldwide has been estimated at over three hundred million. From this pool of carriers, hundreds of thousands die annually from the long-term consequences of chronic hepatitis B (cirrhosis and/or hepatocellular carcinoma).

The hepatitis B delta virus is an agent which, during coinfection with HBV, is responsible for an acute fulminating disease with a generally fatal resolution. The delta virus does not encode (from its own genetic material) proteins which serve as the virion envelope; rather, the virus encapsidates with the envelope proteins encoded by the coinfecting HBV, thereby sharing a close structural and immunologic relationship with the HBV proteins which are described below. It is unknown at this time whether other infectious agents share similar relationships with HBV. However, it is clear that proteins with expanded breadth of serologic reactivity or enhanced immunogenic potency would be useful in systems for diagnosis or prevention (or treatment) of diseases (or infections) by a class of agents with even slight or partial antigenic cross-reactivity with HBV.

The HB virion is composed of two groups of structural proteins, the core proteins and the envelope or surface proteins. In addition to being the major surface proteins or the virion, i.e., Dane particle, the envelope proteins also are the major constituents of Australia antigen, or 22 nm particles. These envelope proteins are the translational products of the large viral open

reading frame (ORF) encoding at least 389 amino acids (aa). This ORF is demarcated into three domains, each of which begins with an ATG codon that is capable of functioning as a translational initiation site in vivo. These domains are referred to as preS1 (108 aa), preS2 (55 aa), and S (226 aa) in their respective 5'-3' order in the gene. Thus, these domains define three polypeptides referred to as S or HBsAg (226 aa), preS2+S (281 aa), and preS1+preS2+S (389 aa), also referred to as p24/gp27, p30/gp33/gp36 and p39/gp42 respectively (as well as the major, middle and large proteins).

Of the two types of structural proteins in the hepatitis B (HB) virion, [envelope or surface ("S") protein and the core ("C") protein], the core protein is the structural protein of the virion nucleocapsid. This "C" protein (HBcAg) is the product of an open reading frame (ORF) of 636 nucleotides and is expressed as intracellular particles as well as on the membranes of infected hepatocytes. The "C" protein ORF is demarcated into two regions, each of which begins with an ATG codon capable of translational initiation in vivo. The presence in virion genomic DNA of a nick at base 8 downstream from the first ATG makes initiation of transcription from the first ATG an improbable event and results in the major synthesized product found in vivo being that initiated from the second ATG. These domains are referred to as Pre-C (87 nucleotides) and C (549 nucleotides) in their 5'to 3' order in the ORF. The product of the C gene (183 amino acids) encompasses all major epitopes of both the

nucleocapsid and core particle.

The envelope proteins of HBV are glycoproteins with carbohydrate side chains (glycans) attached by N-glycosidic linkages to defined peptide recognition sites. [Heermann et al., J. Virol. 52, 396 (1984) and Stibbe et al., J. Virol. 46, 626 (1983)]. O-linked glycosylation may also exist but these sites of derivitization, if they occur, are not clearly known. Thus, the HBV envelope polypeptides produced during natural infection comprise the species p24/gp27 (the S polypeptide and its N-glycosylated derivative), gp33/gp36 (the preS2+S polypeptide N-glycosylated in the preS2 domain only and the same polypeptide N-glycosylated in the S as well as the preS2 domain), and p39/gp42 (the preS1+preS2+S peptide and its derivative N-glycosylated in the preS1 domain). Currently available plasma-derived vaccines are composed of proteins containing virtually only the S domain (comprising the p24 monomer and its N-glycosylated derivative gp27), while yeast-derived vaccines successfully developed to date are composed exclusively of the S polypeptide (comprising exclusively the nonglycosylated p24 species). HBcAg is not glycosylated.

The 22 nm HBsAg particles, have been purified from the plasma of chronic carriers. In terms of their plasma being particle-positive, these chronic carriers are referred to as HBs^+. If infected persons have mounted a sufficient immune response, they can clear the infection and become HBs^-. In terms of their formation of antibodies to HBs, these individuals are denoted anti-HBs^+. In

this way, anti-HBs$^+$ is correlated with recovery from disease and with immunity to reinfection from disease and with immunity to reinfection with HBV. Therefore, the stimulation or formation of anti-HBs by HB vaccines has been expected to confer protection against HBV infection.

This hypothesis has been testable experimentally. Outside of man, the chimpanzee is one of the few species which is fully susceptible to HBV infection , as reflected in quantifiable markers such as HBs$^+$ and elevated serum levels of liver enzyme. Chimpanzees have been vaccinated with three doses of purified HBsAg particles and then challenged intravenously with infectious HBV. While mock-vaccinated animals have shown signs of acute HBV infection, the HBsAg-vaccinated animals have been protected completely from signs of infection. Therefore, in this experimental system, HBsAg particles, composed of p24 (or p24 and gp27), have been sufficient to induce protective immunity. Spurred by these observations, several manufacturers have produced HB vaccines composed of HBsAg particles.

Recently, several independent lines of evidence have suggested that the preS sequences may be important in conferring immunity to HBV. The immune elimination of preS antigens during the course of viral infection appears prognostic for viral clearance and abrogation of infection [Budkowska et al., Ann. Inst. Past./Immun., 136D:56-65, (1985)]. During acute hepatitis B infection, antibodies to the preS domains often arise earlier than antibodies to S [Petit et al., Mol. Immun., 23:511-523, (1986)]. In

inbred mice, the immune responses to S and preS appear to be regulated independently, and the presence of the preS domain can influence the immune response to S [Milich et al., Proc. Nat. Acad. Sci. USA, 82:8168-8172, (1985), J. Immunol., 137:315-322 (1986); Neurath et al., J. Med. Virol., 17:119-125, (1985)]. Furthermore, antibodies to the preS domain neutralize viral infectivity in vitro [Neurath et al., Vaccine, 4:35-37, (1986)], and preS antigens protect immunized chimpanzees against HBV infection [Itoh et al., Proc. Nat. Acad. Sci. USA, 83:9174-9178, (1986)]. In light of these observations and because, as discussed below, of the utility of recombinant yeast in producing HB vaccines from recombinant Saccharomyces cerevisiae, we have formulated experimental HB vaccines from recombinant S. cerevisiae.

In order to expand the available supply of HB vaccines, manufacturers have turned to recombinant DNA technology to mediate the expression of viral envelope proteins. Among microbial systems, Escherichia coli and S. cerevisiae have been used most commonly for the expression of many recombinant-derived proteins. Numerous attempts to express immunologically active HBsAg particles in E. coli have been unsuccessful. However, S. cerevisiae has shown great versatility in its ability to express immunologically active HBsAg particles. These particles (composed exclusively of p24), when formulated into a vaccine, have proven capable of fully protecting chimpanzees against challenge with live HBV of diverse serotypes. Furthermore,

yeast-derived S particles are also immunologically active and as effective in preventing disease or infection in human clinical trials as plasma-derived HBsAg [Stevens et al., JAMA, 257:2612-2616 (1987)]. Therefore, the utility of S. cerevisiae as a host species for directing the synthesis of recombinant HBsAg is established firmly. In addition, expression of human therapeutic agents and vaccines in yeast can be very useful for product development, since yeast is free of endotoxin, is nonpathogenic to man, can be fermented to industrial scale, and lacks many of the safety concerns which surround the use of continuous mammalian cell lines (many of which are virally transformed, may be tumorigenic in mice and all of which contain protooncogenes).

S. cerevisiae (bakers' yeast) is a eukaryote which is capable of synthesizing glycoproteins. Protein glycosylation in yeast has been the subject of numerous recent review articles [notably: Kukuruzinska et al., Ann. Rev. Biochem., (1987) 56, 915-44; Tannen et al., BBA, (1987) 906, 81-99]. This glycosylation or addition of glycans to appropriate receptor amino acids (aa) on the polypeptide occurs either at specific serine (Ser) or threonine (Thr) residues (O-linked) or at specified asparagine (Asn) residues (N-linked). The specificity for O-linked addition at Ser or Thr residues is not clearly understood and is determined empirically on a case-by-case basis.

The signal sequence for N-linked glycosylation is well defined as either of the amino acid sequences Asn-X-Thr or Asn-X-Ser (wherein X is any amino acid). In addition to synthesizing many autologous, native, glycosylated proteins (among them

being those called mannoproteins, or mannopeptides), yeast also are capable of glycosylating heterologous or foreign proteins expressed by recombinant technology (if the heterologous protein contains the appropriate glycosylation signal sequence for either N-linked or O-linked glycosylation, although all potential sites may not be glycosylated).

The preS2+S polypeptides, which are produced during natural infection contain no more than two [ca. 3 kilodaltons (kD) in size] N-linked glycans, one in the S region and a second on the Asn at amino acid residue 4 of the preS2 domain. The recognition site in the S domain is not glycosylated in either Recombivax HB® or in recombinant preS2+S synthesized in yeast. However, the site at amino acid residue 4 of the preS2 domain is recognized and glycosylated by yeast.

The preS1 domain contains an N-linked glycosylation site at amino acid residue 4 of the preS1 region and a potential site at aa residue 26 for serotype adw. It is readily apparent to those skilled in the art that arguments set forth for preS2 glycosylation also will follow for diverse sequences in the preS2 region as well as for those in the preS1 and S domains.

Yeast synthesizing recombinant preS1+preS2+S or preS2+S add a glycan which is similar to that added to the native polypeptide during viral infection. However, if the yeast host cell is "wild-type" for glycosylation (i.e., containing the full complement of enzymes required for native glycosylation which is the case for virtually all commonly used yeast strains), a significant number of

these glycans are extended with a large number of additional mannose residues in a manner identical to that employed by yeast in making its own structural mannoproteins. This extended addition of the glycan, when it occurs on a foreign gene product such as the preS1+preS2+S or preS2+S polypeptide, is referred to as hyperglycosylation or hypermannosylation. It is readily apparent to those skilled in the art that arguments set forth for yeast also will extend to other host cells (e.g., insect, fungi, etc.) which may be subject to divergent glycosylation patterns.

Furthermore, it has been demonstrated that recombinant forms of 22nm particles of HBV surface proteins expressed in wild-type yeast host cells, may entrap substantial amounts of yeast cell carbohydrate (deriving at least in part from the structural mannoproteins and mannopeptides of the yeast host cell) within the 22nm particle. This entrapped carbohydrate may cause the generation of antibodies against yeast carbohydrate moieties on glycosylated proteins, and a vaccine immunogen containing entrapped yeast carbohydrate would react with anti-yeast antibodies present in most mammalian species thereby potentially diminishing its effectiveness as an immunogen and vaccine.

Hyperglycosylation and entrapment of complete mannoproteins and mannopeptides may be eliminated or glycosylation limited in HBV preS and S containing polypeptides and their corresponding particles by any of the following approaches.

Firstly, N-linked hyperglycosylation may be

prevented or limited during growth of the recombinant host through the presence in the growth medium of an exogenous agent (e.g., tunicamycin). Secondly, polypeptides, from recombinant or natural sources may be deglycosylated either chemically (e.g. anhydrous trifluoromethane- sulfonic acid or anhydrous hydrogen fluoride) or enzymatically (e.g., with N-glycanase, Endo-F or Endo-H) or physically (e.g. sonication). Thirdly, the recognition site for N-linked glycosylation may be changed or deleted by mutagenesis at the DNA level, such that all N-linked glycosylation, and thereby hyperglycosylation as well, is prevented. Such modified preS+S ORF's in which the glycosylation recognition sequence has been altered (directed by suitable promoters active in yeast) have been transformed into yeast host cells. The resultant preS+S polypeptides lack N-linked glycosylation. Fourthly, host cells may be identified which lack critical enzymes required for glycosylation, which, in part, illustrates the present invention without however limiting the same thereto. One such yeast strain has been identified (mnn^9- mutant) [Ballou, L et al., (1980), J.Biol.Chem., 255, pp 5986-5991] which lacks a critical enzyme in the glycosylation pathway necessary for the elongation (hyperglycosylation) of the N-linked glycans; chemical studies indicate that this mutant makes mannoproteins without outer-chain mannose residues and containing only the inner chain carbohydrate [Ballou, C.E. et al., (1986), Proc.Natl.Acad.Sci.U.S.A., 83, pp 3081-3085; Tsai, P. et al., (1984), J.Biol.Chem., 259, pp 3805-3811]. The ORF for the S and/or preS+S polypeptide

(transcription directed by suitable promoters active in yeast) has been used to transform such mnn9- mutant yeast. The resulting preS+S polypeptide (including preS1+preS2+S, preS2+S, preS1, preS2, or preS1+preS2) contains only inner chain N-glycosylation and lacks hyperglycosylation. If the N-glycosylation signal is mutated to change Asn-4 of the preS1 or the preS2 domains to Gln-4, the resulting polypeptides are not glycosylated.

Although the HBV polypeptides are neither glycosylated nor hyperglycosylated when expressed in these mutant yeast, particles composed therefrom may contain significant levels of entrapped carbohydrate deriving from yeast mannopeptides. Therefore, the expression of S polypeptides as well as preS containing polypeptides in yeast cells which cannot hyperglycosylate results in decreased levels of carbohydrate in the expressed 22nm particles.

The potential benefit which may derive from inclusion of HBcAg in HB vaccines as yet is unsubstantiated clinically. Although anti-HBc is present in acute hepatitis B and persists during chronic hepatitis B without disease resolution, and although anti-HBc antibody is transferred transplancentally but does not prevent perinatal infection of newborns from carrier mothers, there is some persuasive experimental evidence that HBcAg should be re-evaluated as at least one component of future vaccines. In fact, immunization of chimpanzees with HBcAg can protect from or modify HBV infectivity (Murray et al., 1984, EMBO J., 3, pp.645-50 and Iwarson et al., 1985, Gastroenterology, 88, pp.763-7). Furthermore, HBcAg can induce cell

mediated immune (CMI) responses in both laboratory animals and humans; HBsAg is much less effective in eliciting CMI (Gerety et al., 1974, J. Immunol., 113, pp.1223-9). Recombinant HBcAg has been expressed and purified successfully in several laboratories (Pasek et al., 1985, Nature, 282, pp.575-9, Kniskern et al., 1986, Gene, 46, pp.135-41 and Roossinck et al., 1986, Mol. Cell. Biol. 6, pp.1393-4000) and such recombinant HBcAg forms 28-nm particles which are virtually indistinguishable from the native antigen isolated from the livers of infected individuals (Petit et al., 1985, J. Virol., 52, pp.543-51). However, HBcAg is a nucleic acid binding protein, such that the recombinant particles, like their native counterparts, contain nucleic acid at levels well above that proposed to be acceptable according to guidelines for recombinant vaccines (CBER, April 10, 1985). The removal of the C-terminal nucleic acid binding domain to produce a version of the antigen which might retain the desired immunogenic properties, presents a lead which may overcome this obstacle. The present invention also illustrates a procedure to remove nucleic acid from recombinant particles to levels acceptable for human vaccine use. In addition, experimental evidence exists which demonstrates by co-administration of virions containing HBcAg along with the envelope proteins (Milich et al., supra) an induction of anti-HBs and anti-PreS responses in mice, otherwise genetically incapable of making such antibodies. Such combined vaccines, in the form of mixed particles, may hold promise for overcoming genetically linked

non-responsiveness in humans, and are the subject of the present invention.

S. cerevisiae has shown great versatility in its ability to express immunologically active 22 nm particles. These particles, when formulated into a vaccine, have proven capable of fully protecting chimpanzees against challenge with live HBV. Furthermore, yeast-derived HBsAg has been effective immunologically in human clinical trails as plasma-derived HBsAg. Therefore, the utility of S. cerevisiae as a host species for directing synthesis of recombinant HBsAg is established firmly.

In a variety of recombinant microbial expression systems, the synthesis of many different polypeptides has been shown to be deleterious to the host cell. As a consequence, there is selective pressure against the expression of such polypeptides, such that the cells which may accumulate in a scale-up of such a recombinant culture are those which have ceased to express the foreign polypeptide or express so little of the foreign polypeptide that the culture becomes an uneconomical source of that polypeptide. In some cases, the deleterious effect is so strong that when expression is driven by a strong constitutive promoter, newly transformed cells fail to propagate and form colonies on selective plates. These deleterious effects can be overcome by using a regulatable promoter to direct the synthesis of such polypeptides. A number of regulatable or inducible genes exist in S. cerevisiae. Four well-characterized inducible genetic systems are the galactose (GAL) utilization genes, the alcohol

dehydrogenase 2 (ADH2) gene, the alpha mating factor gene, and the pho5 gene.

S. cerevisiae has 5 genes which encode the enzymes responsible for the utilization of galactose as a carbon source for growth. The GAL1, GAL2, GAL5, GAL7 and GAL10 genes respectively encode galactokinase, galactose permease, the major isozyme of phosphoglucomutase, α-D-galactose-1-phosphate uridyltransferase and uridine diphospho-galactose-4-epimerase. In the presence of glucose and the absence of galactose, very little expression of these enzymes is detected. If cells are grown on glucose and then galactose is added to the culture after glucose depletion, these three enzymes are induced coordinately, by at least 1,000-fold, (except for GAL5, which is induced to about 5 fold) at the level of RNA transcription. The GAL1 GAL2, GAL5, GAL7 and GAL10 genes have been molecularly cloned and sequenced. The regulatory and promoter sequences to the 5' sides of the respective coding regions have been placed adjacent to the coding regions of the lacZ gene. These experiments have defined those promoter and regulatory sequences which are necessary and sufficient for galactose induction.

S. cerevisiae also has 3 genes, each of which encodes an isozyme of ADH. One of these enzymes, ADHII, is responsible for the ability of S. cerevisiae to utilize ethanol as a carbon source during oxidative growth. Expression of the ADH2 gene, which encodes the ADHII isozyme, is catabolite-repressed by glucose, such that there is virtually no transcription of the ADH2 gene during fermentative

growth in the presence of glucose levels of 0.1% (w/v). Upon glucose depletion and in the presence of non-repressing carbon sources, transcription of the ADH2 gene is induced 100- to 1000-fold. This gene has been molecularly cloned and sequenced, and those regulatory and promoter sequences which are necessary and sufficient for derepression of transcription have been defined.

Alpha mating factor is a sex pheromone of S. cerevisiae which is required for mating between MATα and MATa cells. This tridecapeptide is expressed as a prepropheromone which is directed into the rough endoplasmic reticulum, glycosylated and proteolytically processed to its final mature form which is secreted from cells. This biochemical pathway has been exploited as an expression strategy for foreign polypeptides. The alpha mating factor gene has been molecularly cloned and its promoter with pre-pro-leader sequence has been utilized to express and secrete a variety of polypeptides. Likewise, the pho5 gene promoter has been shown to be inducible by low phosphate concentrations and thus also has utility for physiologically regulated expression of foreign proteins in yeast.

As expected by their traversal of the rough endoplasmic reticulum and Golgi apparatus, foreign proteins can undergo both N- and O-linked glycosylation events. The alpha mating factor promoter is active only in cells which are phenotypically α. There are 4 genetic loci in S. cerevisiae, known as SIR, which synthesize proteins required for the repression of other normally silent

copies of a and α information. Temperature-sensitive (ts) lesions which interfere with this repression event exist in the gene product of at least one of these loci. In this mutant, growth at 35°C abrogates repression, resulting in cells phenotypically a/α in which the alpha mating factor promoter is inactive. Upon temperature shift to 23°C, the cells phenotypically revert to α such that the promoter becomes active. The use of strains with a ts SIR lesion has been demonstrated for the controlled expression of several foreign polypeptides.

Accordingly, the present invention provides multiple HBV surface proteins and HBV core protein simultaneously expressed in a yeast host which form mixed particles composed of two or more HBV proteins. In addition, the present invention provides a method of producing in a yeast host, multiple HBV surface proteins which form particles also containing HBcAg and which contain substantially reduced entrapped carbohydrate content. The present invention also provides a vaccine against HBV comprising the HBV protein particle containing multiple HBV surface proteins and HBV core protein (in the same particle) with substantially reduced entrapped carbohydrate and nucleic acid content for both active and passive treatment of prevention of disease and/or infections caused by HBV or other agents serologically related to HBV. Also provided are antigens and immunogens for the development of diagnostic reagents for such diseases and/or infections. Further the present invention provides conditions for the large scale growth of recombinant host cells and the purification

of the recombinant multiple HBV surface proteins plus HBV core protein in particulate form.

SUMMARY OF THE INVENTION

Multiple HBV proteins have been simultaneously expressed at high yield and form particles in a recombinant yeast host. The expression of multiple HBV surface proteins simultaneously in yeast cells results in the formation of the characteristic particles containing (within the same particle) multiple forms of the proteins at predetermined ratios. Formation of these particles in a "wild-type" yeast host cell may result in the entrapment of yeast cell substances within the particles. Using "wild-type" yeast host cells substantial amounts of yeast cell carbohydrate may become entrapped within the HBsAg particles. In order to circumvent the production of a HBV vaccine consisting of particles which contain substantial amounts of carbohydrate, the HBV surface proteins may be simultaneously produced and purified from a recombinant yeast host which is genetically deficient in its ability to glycosylate proteins. Multiple HBV surface proteins produced by such a host form particles which contain substantially less carbohydrate than particles produced in wild-type yeast cells. The use of truncated forms of HBcAg or procedures to remove nucleic acids from recombinantly expressed core-containing particles results in a vaccine also containing nucleic acid levels below those thought to be acceptable for human vaccine

use. These HBV protein particles, containing multiple surface and core proteins at various ratios and if produced in glycosylation deficient yeast also having substantially reduced entrapped carbohydrate content and nucleic acid content, are useful as a vaccine for the active or passive treatment and/or prevention of HBV related infections, for preparation of diagnostic or therapeutic antisera and antibodies, and as an antigen for immunologic diagnosis with reduced reactivity with naturally occuring anti-yeast antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 - Figure 1 is a schematic diagram of cloning of plasmid pYGC-1 which contains the HBcAg ORF.

Figure 2 - Figure 2 is a schematic diagram of plasmid pKHBS-5a which contains the HBcAg ORF, the pADH2 promoter, the tADH1 terminator and a LEU2+ selectable marker in a pC1/1 based plasmid.

Figure 3 - Figure 3 is a schematic diagram of plasmid pKHBS-2a which contains the preS1+preS2+S ORF, the pGAL10 promoter, the tADH1 terminator and a URA selectable marker in a pYEP24 based plasmid.

Figure 4 - Figure 4 is a schematic diagram of plasmid pKHBS-5b which is the bidirectional promoter vector containing the preS1+preS2+S ORF driven by the pGAL10 promoter, and the HBcAg ORF driven by the pGAL1 promoter in a pC1/1 based plasmid.

Figure 5 - Figure 5 (a) is an electron micrograph of yeast-produced core-containing mixed particles.

Figure 5 (b) is an electron micrograph of yeast produced core containing mixed particles treated with a preS2-specific monoclonal antibody.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a method for the preparation of one or more HBV proteins, one of which is HBcAg. In the case of multiple HBV proteins, the proteins form mixed particles containing two or more proteins in the same particle, for use as a vaccine against HBV. In addition, the invention is directed to such particles containing substanitally reduced carbohydrate and nucleic acid content. Therefore, expression was achieved in yeast hosts which contain the mnn9- mutation which prevents hyperglycosylation and co-purified of entrapped yeast carbohydrate as well as in yeast cells which are wild-type for glycosylation. In addition, because yeast N-glycosylate the preS1 domain at ASN4, this residue was mutated to GLN (Q4) in the preS1+preS2+S ORF to completely eliminate N-linked glycosylation, at that site, in the expressed polypeptides. Other potential, N-linked glycosylation sites, one in the preS2 domain and two in the S domain, are not glycosylated by most yeast strains. It is readily apparent to those skilled in the art that mutated forms of the protein at these sites are encompassed by the present invention for yeast or other

eukaryotes capable of such glycosylation. It is also readily apparent to those skilled in the art that non-mutated forms of the protein are encompassed by this invention, and are described below. In order to develop an HBV vaccine candidate for protection of individuals who are hypo- or non-responsive to current vaccines by expressing a mixed-particle antigen displaying PreS1 (and PreS2) epitopes on HBsAg and HBcAg particles which also display the protective "a" epitope of HBsAg and the T cell and B cell immunologic epitopes of HBcAg, three interrelated strategies were developed.

One strategy involves co-expressing the preS1(Q4)+preS2+S gene with the core gene, to produce a framework of particles formed of S antigen and core antigen into which the preS1(Q4)+preS2+S protein is inserted. Co-expression is achieved in three different ways. First, expression is achieved by transforming one yeast cell with two separate autonomously replicating, independently selectable plasmids (double transformants) each carrying one HBV gene. Based on approximate expected relative plasmid copy numbers, the ratio of the expressed polypeptides in this transformant is about 10:1 (C:preS1(Q4)+preS2+S). Second, this was achieved by putting two HBV genes on one plasmid under the control of two separate promoters in a bidirectional vector. Based on approximate expected relative promoter strengths the ratio of the expressed polypeptides in this transformant is about 1:1 (C:preS1(Q4)+preS2+S). Third, this was achieved by putting two HBV genes under control of two separate

promoters in a two cassette vector construction. Based on approximate expected relative promoter strengths, the ratio of expressed polypeptides in this transformant is about 3:1 or 1:1 (C:preS1+preS2+S) depending on yeast host phenotype. These strategies all have been successful and the transformed yeast express both HBV polypeptides in different apparent ratios. The polypeptides co-assemble into particles which display the preS domain as well as the protective a epitope of HBsAg, and the HBcAg.

Dane particles (serotype adw or ayw) were utilized as the source of HBV nucleic acid for the isolation of the viral ORFs. It is readily apparent to those skilled in the art that this invention extends to the use of nucleic acid from HBV strains or related viruses with other serologic reactivities (including but not limited to ayr, ayw, adr, adw and other a epitope variants) which derive from viral genetic diversity. The endogenous polymerase reaction was employed in order to produce covalently-closed circular double-stranded DNA of the HBV genome from the nicked and gapped nucleic acid form that natively resides in the HB virion. The DNA was isolated, digested to completion with EcoRI, and cloned into the EcoRI site of pBR322, thus generating pHBV/ADW-1, or pHBV/AYW-1. The recombinant plasmids containing the HBV genome in a circularly permuted form at the EcoRI site of the PreS region were selected.

The HBcAg gene is isolated from such recombinant pBR322 by digestion with the restriction enzymes, HhaI and AvaI, isolation of the 0.6 kilobase-pair (kbp) fragment, and digestion with the

enzyme, TaqI, to yield a 0.5 kbp DNA fragment isolated by preparative agarose gel electrophoresis with TaqI and AvaI termini. The missing bases at the 5' and 3' ends of the HBcAg are reconstituted to the original composition with the appropriate synthetic oligonucleotides, including the optimized nontranslated leader at the 5' end, yielding a 0.57 kbp fragment with the restriction enzyme, HindIII termini containing an optimized nontranslated leader (NTL) sequence and the complete HBcAg ORF.

The expression of HBcAg in yeast species is done using a plasmid expression vector containing yeast-derived sequences for the selection and amplification of the plasmid, yeast promoter, an optimized 5' nontranslated leader having the nucleotide sequence ACAAAACAAA (SEQ.ID.NO.: 1), the HBcAg coding region and the yeast transcriptional termination sequence. It is readily apparent to those skilled in the art that the present invention encompasses other 5' and 3' nontranslated flanking sequences. The 3' end of the yeast promoter is directly linked to the 5' end of the optimized nontranslated leader, and the 3' end of the optimized nontranslated leader is directly linked to the initiation codon of the HBcAg coding region.

The complete ORF encoding the 108 amino acids of the preS1 region, the 55 amino acids of the preS2 region, and the 226 amino acids of the S region was constructed as follows: The plasmid (pBR322 above) contains the HBV genome in a circularly permuted form in which the EcoRI site divides the complete preS1+preS2+S coding region into a 5' domain of 0.4 kilobase pairs (kbp) and a 3' domain of 0.8

kbp. These two domains are subcloned for the eventual reassembly of the entire gene. For the 3' domain, pUC19 is digested with EcoRI and BamHI, then ligated to a synthetic oligonucleotide which consists of the final 5 nucleotides of the coding region, the stop codon, a HindIII site, and a BamHI end. The 3' portion of the preS1+preS2+S gene, consisting of a 0.8 EcoRI-AccI fragment, is cloned into this vector. The 5' portion, consisting of a 0.3 kbp BamHI-EcoRI fragment, is subcloned into pUC18 in either of two ways, depending upon whether (1) the complete ORF is to be expressed or (2) the putative hydrophobic signal sequence (amino acids 2-15) is to be eliminated. For the first stragegy, pUC18 is digested with HindIII and EcoRI and ligated to a 72 bp synthetic oligonucleotide which reconstitutes the complete ORF from the BamHI site upstream, through the distal ATG and a 10 bp nontranslated leader sequence, to a HindIII compatible terminus. For the second strategy, a 30 bp oligonucleotide is ligated to the above vector which performs an identical function but which eliminates the coding region for amino acids 2-15. The 0.3 kbp BamHI-EcoRI fragment of the 5' domain then is ligated into either of these oligonucleotide-linked cloning vectors. The 5' pUC18 and 3' pUC19 clones are amplified by growth in E. coli, and the coding regions are digested from the isolated plasmids as HindIII-EcoRI fragments. The 5' and 3' fragments are ligated, digested with HindIII, and the complete ORF with HindIII termini is cloned into pUC13 which had been digested previously with HindIII. The complete ORF as a HindIII fragment is available in this vector.

An Asn4 to Gln4 mutation was made in the preS1+preS2+S ORF by insertion of an appropriate synthetic oligonucleotide. The ORF was inserted into the pGAL10 expression cassette and the oriented cassette was ligated into the two yeast shuttle vectors described above (pC1/1, and YEp24).

The expression cassette (pGAL10-tADH1) drives expression of foreign genes inserted at a unique HindIII site downstream from the galactose-inducible GAL10 promoter. The preS1+preS2+S ORF (with HindIII termini) described above was ligated into the HindIII site of the vector. This expression cassette was inserted between the SphI sites of the E. coli/S. cerevisiae shuttle vector pC1/1 (Beggs, supra) and this vector was introduced into S. cerevisiae strains CF52 or CF55 and transformed clones were selected.

The fragment encoding either C, or preS1+preS2+S described above was used to construct an expression cassette, as described previously [Kniskern et al., Gene 46:135-141, (1986)], which was composed of: (a) ca. 1.1 kbp of the GAP491 promoter, (b) a 10 bp yeast-derived flanking sequence, (c) 1230bp of the viral ORF for preS1+preS2+S, or 550 bp of the viral ORF for C, and (d) about 0.4 kbp of the yeast ADH1 terminator.

Three different expression vectors were used to construct the HBV protein expression cassettes. The GAP 491 promoter expression cassette described previously [Kniskern et al., 1986 Gene 46 pp135-141], is composed of about 1.1 kbp of the

glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) promoter and about 350bp of the yeast alcohol dehydrogenase 1 (ADH1) terminator in a pBR322 backbone, with a unique HindIII site between the promoter and terminator. The DNA ORFs were ligated in the unique HindIII site, and its presence and orientation confirmed by restriction endonuclease analyses and Southern blot.

Alternately the (0.5kbp) GAL10 promoter (Schultz et al., 1987, Gene, 54, pp113-123) was substituted for the 1.1kbp GAP promoter in the above construction, or the (1.25 kbp) ADH2 promoter (Kniskern et al., 1988 Hepatology 8, 82-87) was substituted for the GAP promoter.

In each case, the expression cassette containing the specific promoter, the HBV protein ORF, and the ADH1 terminator were cloned into a shuttle vector [pC1/1 (Beggs, supra; Rosenberg, et al., supra) or YEp24 (New England Biolabs)] to create a yeast expression vector which was then used to transform, or cotransform with a second vector, S. cerevisiae as described below. These transformants were established as frozen stocks for evaluation and subsequent experimentation.

Parental strains CF52 or CF55 were obtained as follows: The α mating type strain CZ5/LB347-1C (mnn9$^-$, SUCZ$^-$) was mated with the "a" type strain 2150-2-3 (leu2$^-$, ade1$^-$) by mixing the strains on a YEHD complete media plate. To select for diploids, the mated strains were replica plated onto leu$^-$ minimal medium containing 2% sucrose as the sole carbon source. After isolating single colonies, the diploids were sporulated, and asci were dissected by

standard techniques. The KHY-107 strain was isolated as a single spore and characterized as cir$^+$, ade1$^+$, leu2$^-$, and mnn9$^-$ (by Schiff stain technique). KHY107 (cir 0) was derived from strain KHY107 (cir$^+$) as described by Broach [Methods in Enzymology, 101, Part C, pp 307-325, (1983)]. The cured strain was made ura3$^-$ by integrating a disrupted ura3 gene. The resulting strain, KHY-107ura3Δ, was grown in rich media to allow the accumulation of spontaneous mutations and a canavanine resistant mutant was selected. The mutant strain, CF55, was shown by complementation tests to be can1$^-$. The GAL10pGAL4 expression cassette was integrated into the HIS3 gene of CF55 (Methods in Enzymology, 1990, 185 pp297-309) to yield the final host strain CF52 (Mata leu2-2,112 ura3Δ can1 his3Δ::GAL10pGAL4-URA3, cir° mnn9$^-$). The strains CF52 and CF55 were established as frozen stocks for evaluation and subsequent experimentation.

A yeast active promoter initiates transcription of the HBsAg, HBcAg and related genes. Therefore, it is readily apparent to those skilled in the art that any yeast-active promoter sequence (e.g. including by not limited to GAL1, GAL10, ADH2 Pho5, etc.) may be substituted for the GAP491 promoter. It is also readily apparent to those skilled in the art that a suitable assay system, e.g., immunoblot or RIA or enzyme-linked immunoassay (EIA), should be utilized in order to assay expression of HBcAg, HBsAg and related polypeptides in this system, such that the time of harvesting of the culture for attaining a maximal yield can be optimized.

The GAP491 promoter has been useful for the expression in yeast of several foreign proteins, including HBsAg [Bitter et al., Gene, 32:263-274, (1984); Wampler et al., Proc. Nat. Acad. Sci. USA, 82,pp. 6830-6834, (1985)]. Based upon our previous results of expressing HBcAg to about 40% of soluble yeast protein (Kniskern et al., supra), we have used this promoter to drive the expression of HBsAg and related proteins in suitable yeast host cells.

It is readily apparent to those skilled in the art that the selection of a suitable yeast strain for expression of HBsAg and HBcAg encompasses a wide variety of candidates. Suitable yeast strains include but are not limited to those with genetic and phenotypic characteristics such as protease deficiencies, and altered glycosylation capabilities.

In order to control and define the glycosylation of recombinant yeast-expressed HBV proteins, S. cerevisiae strain CF52 (Mata leu2-2, 112 ura3Δ can1 his3Δ:: GAL10pGAL4-URA3, cir°, mnn9-) was employed. CF55 (Mata leu2-2,112 ura3Δ can1 cir° mnn9-) which was constructed as described above, was employed for double transformants which require a host lacking ura3 as well as the leu2 gene.

The expression plasmid pC1/1pADH2-HBcAg-tADH-1 was used either alone or with a second plasmid to transform CF52 (Mata leu2-2, 112 ura3Δ can1 his3Δ:: GAL10pGAL4-URA3, cir°, mnn9$^-$) or CF55 (Mata leu2-2,112 ura3Δ can1 cir° mnn9$^-$). Transformed clones were selected on minimal medium (leu-, and also ura- for two vector transformants of CF55) containing 1M sorbitol. These cloned transformants

were established as frozen stocks in 17% glycerol for subsequent evaluation and further experimentation.

To produce a glycosylation wild-type control the expression plasmids were also used to transform yeast strain CF54, which was isolated by standard techniques from strain CF52 and which is a spontaneous revertant to MNN9+ (and thus is wild-type for glycosylation, but otherwise of identical genotype to strain CF52). Transformed clonal isolates were established as frozen stocks in 17% glycerol for subsequent evaluation and further experimentation.

Clones of transformed yeast containing the expression plasmids were plated onto leu$^-$ selective agar plates (containing 1M sorbitol for mnn9- transformants, and also lacking uracil for two vector transformants of CF55) and incubated at 30°C for 2-3 days. These yeast were inoculated into 5-7 mL cultures of complex YEHD (Carty et al., supra) medium (containing 0.1M to 1M sorbitol) and the cultures were incubated at 30°C with aeration for 12-18 hours. Flasks containing 50 mL complex YEHD media with 1M sorbitol (hereafter called YEHDS), plus 2% galactose for GAL10 promoter plasmids, were inoculated from the above cultures (to an inital A_{600} = 0.1) and were incubated at 30°C with shaking (350 rpm) for 48-72 hours to a final A_{600} of 10-16. Samples of 10 A_{600} units were dispensed into tubes, and the yeast cells were pelleted by centrifugation at about 2000xg for about 10 minutes. Samples either were assayed directly or stored frozen at -70°C. At the time of assay, the pellets were resuspended in

0.4 mL of phosphate buffered saline (PBS) containing 2mM phenylmethyl sulfonyl fluoride (PMSF) and transferred to 1.5 mL Eppendorf tubes. Yeast cells were broken by: 1) the addition of 200-300 mg of washed glass beads (0.45 mm) and agitation on a vortex mixer for 15 minutes, 2) addition of TRITON X-100 to 0.5%, 3) agitation on the vortex mixer for 2 minutes, and 4) incubation at 4°C for 10 minutes. Cellular debris and glass beads were removed and the supernatant assayed for protein [by the method of Lowry et al., J. Biol. Chem., 193, 265, (1951)] and RIA specific for preS2+S [Hansson et al., Infect. Immunol. 26: 125-130, (1979), Machida et al., Gastroenterology 86: 910-918, (1984)], or S (AUSRIA).

Immunoblot analysis of the polypeptide derived from all recombinant clones described above, showed two bands with apparent molecular size of about 19-kD (for C), and about 41kD (for the preS1(Q4)+preS2+S).

For recombinant proteins, the qualitative and quantitative glycosylation patterns are a function of and largely dependent upon the host cell species, and within a species upon the cell line. It is thus readily apparent to those skilled in the art that the selection of a host strain extends to species and cell lines other than S. cerevisiae for which mutations in enzymes in the glycosylation pathway may be identified. It is also readily apparent to those skilled in the art that selection of host strains of S. cerevisiae extends to all strains in which mutations in enzymes of the glycosylation pathway may be identifed.

The transformed clones were then screened for the presence of the preS1+preS2+S DNA and HBcAg DNA, and expression of p19 and p41. Cells were grown in YEHDS medium also containing galactose for the GAL10 promoter plasmids to induce expression following glucose depletion. Lysates were prepared, resolved by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotted to nitrocellulose. Both a p19 and a p41 product were found to be specific HBV proteins by virtue of their presence only in induced transformants and their reactivity with antiserum. The p19 band was reactive only with anti-HBc and p41 was reactive with either anti-preS (preS1 or preS2) or anti-p24(S) antisera. One clone of each strategy was selected for further analysis. Furthermore, lysates of transformants, but no parental S. cerevisiae, were positive for HBcAg, HBsAg and preS, by radioimmunoassay.

This highlights the utility of the expression vectors which utilizes the GAL10 promoter to direct the expression of HBsAg and related HBV proteins in S. cerevisiae. It is readily apparent to those skilled in the art that the regulatable GAL10 promoter, or GAL1, GAL2, GAL7 or MEL1 promoters which function in an indistinguishable manner, enable the growth of a recombinant S. cerevisiae culture to be scaled up to a production-scale volume before synthesis of the recombinant protein is initiated, such that negative effects on the host cell are minimized. Moreover, it is readily apparent to those skilled in the art that an expression vector containing another regulatory promoter, including but

not limited to ADH2 and alpha mating factor, physiologically inducible or derepressible by other means, can be utilized to direct expression of S, C and preS-containing peptides. Furthermore, it is readily apparent to those skilled in the art that a constitutive promoter less potent than GAPDH, including but not limited to CYC1, drives expression of S and pre-S-containing polypeptides to a lower percentage of cell protein, such that the negative physiological effects of overexpression would be obviated. It is readily apparent to those skilled in the art that a suitable assay system, e.g., Western blot or radioimmunoassay, should be utilized in order to assay expression of S, C and pre-S-containing polypeptides in this system so that the time of harvesting of the culture for attaining a maximal yield can be optimized.

PreS1 and core protein-containing vaccines which form immunogenic particles, i.e., a species of mixed particle of preS1(Q4 or N4)+preS2+S, and core protein retaining the protective "a" epitope and also displaying epitopes of the preS1 (and preS2) region(s), and HBcAg have been developed. Two interrelated strategies are used: (1) double transformants of a single yeast host with two independently selectable, autonomously replicating vectors, one expressing an envelope polypeptide and another expressing the core polypeptide; and (2) transformation of a yeast host with a bi-directional (double) expression vector capable of expression of one envelope polypeptide and the core polypeptide from the same plasmid backbone. Forms of

preS1-containing surface protein that have been used include: (1) the complete preS1+preS2+S ORF which will core glycosylate at Asn4 of the preS1 region in a mnn9- host; (2) an edited (Δ2-15) version of the complete preS1+preS2+S ORF which lacks amino acids 2-15 and thus can be neither glycosylated nor myristylated but which still retains the major antigenic/neutralizing epitope of preS1 or (3) an Asn4 to Gln4 mutant incapable of being N-glycosylated in any host background.

The complete preS1+preS2+S (as well as the Δ2-15 variant and preS1(Q4)+preS2+S) was cloned into the pGAL10 expression cassette, and this cassette was engineered into the Ura-based yeast shuttle vector YEp24, a medium-copy (ca. 30 copies/cell) 2μ-based vector. This vector has been used to double transform several yeast strains along with a high copy number (ca. 200-400 copies/cell) Leu^--based vector (pC1/1) into which was constructed a pADH2 expression cassette expressing the C ORF. Transformants were cloned on double-selection plates lacking both leucine and uracil. Difficulties with low frequency recovery of double transformants were overcome by mildly enriching the recovery medium with nutrients, and clones of transformants with the complete preS1+preS2+S in YEp24 along with C on pC1/1 were positive by AUSRIA (S), preS-RIAs and CORAg (c), all of which require the presence of particles, and the preS RIAs requiring the presence of preS epitopes on AUSRIA-positive or CORAg-positive particles. In addition immunoblot analysis shows a band at about 41 kD (size consistent with the expected translation

product of complete preS1+preS2+S ORF) which was reactive with anti-preS (preS1 and preS2) as well as anti-S antibodies, and a p19 band reactive only with anti-C antibodies. Estimates by immunoblot suggest a relative ratio for p19:p41 of about 10:1. Two clones were grown in larger volume so that particles could be purified by immunoaffinity chromatography. Similar results were obtained with the Δ2-15 ORF.

An Asn4 to Gln4 mutation was made in the preS1+preS2+S ORF by insertion of an appropriate synthetic oligonucleotide. The ORF was inserted into the pGAL10 expression cassette and the oriented cassette was ligated into the two yeast shuttle vectors described above (pC1/1, and YEp24).

An expression cassette which allows the expression of two polypeptides from two GAL-regulated promoters on a single vector backbone was constructed. This construction takes advantage of the bi-directionally divergent yeast pGAL1pGAL10 promoter region. Unique cloning sites between the promoters and added ADH1 sequences were engineered. The C and preS1+preS2+S ORFs were inserted at these unique sites. This bidirectional vector was used to coexpress the ORFs for C and preS1+preS2+S(ay) from pGAL1 and pGAL10 respectively. Yeast were transformed, and cloned seeds established.

A second strategy for expression of two ORFs from the same vector was the use of promoters of different strengths in order to provide for different relative levels of expression of preS1+preS2+S compared to double transformants (1:10) or

bidirectional GAL promoters (1:1). Therefore, the pADH2/C and pGAL10/preS1+preS2+S ORFs were inserted onto pC1/1.

Yeast cells transformed with expression vectors coding for hepatitis B virus proteins or variants thereof are grown and harvested. The cells may be stored if desired by washing the cells in a buffer solution, e.g. PBS, and forming a cell paste which is typically stored frozen at -70°C.

Purification of HBsAg and related proteins typically begins as follows. A batch of fresh or frozen cell paste is suspended in a buffer, preferrably TRIS, at a high pH ranging between about 8.5 and about 11.0, preferrably about 10.5 (the buffer may also contain suitable protease inhibitors). The cells are then disrupted, preferrably by mechanical means. The gentle bead breakage method of disruption has been found to be unsuitable for scale-up use. Disruption by a high pressure homogenizer (about 10,000 to 20,000psi, using a Gaulin or Stansted homogenizer) is preferred because of its rapid and efficient operation.

An immune-affinity column, bound with goat antibodies which recognize the particulate form of HBsAg, has been utilized to purify S and S-related proteins from transformed S. cerevisiae. The eluted product is positive for HBsAg by radioimmunoassay, and is of particulate form in electron microscopy. Such a particulate form which contains both HBsAg and pre-S antigens or HBsAg alone is effective as a HBV vaccine and diagnostic reagent.

The carbohydrate content of the particles is

determined by the method of Dubois, M. et al., Anal. Chem., 28, pp.350, 1956. The general principle of this procedure is that simple sugars, oligosaccharides, polysaccharides and their derivatives, including the methyl ethers with free or potentially free reducing groups, give an orange yellow color when treated with phenol and concentrated sulfuric acid. The amount of color produced at a constant phenol concentration is proportional to the amount of sugar present.

To determine the carbohydrate content of a sample of HBV surface proteins, 1 mL of a solution containing between 10 to 70 µg of protein is placed in a test tube. A series of carbohydrate standards and blank samples are prepared. One mL of a 5% phenol solution is added to each tube, the tubes are mixed, and 5 mL of a 96% sulfuric acid solution is added and mixed. The tubes are incubated at room temperature for 10 minutes, mixed, and incubated at 25 to 30°C for 20 minutes. The samples are read in spectrophotometer (A_{490} for hexoses and methylated hexoses, and A_{480} for pentoses, uronic acid, and their methylated derivatives) and the amount of carbohydrate in the HBV samples is determined by comparison with the carbohydrate standards.

Particles produced in "wild-type" recombinant yeast cells (CF54), and HBV antigens produced in the CF52 recombinant yeast cells were both analyzed for carbohydrate content as described above. Based on these results, a ratio of the amount of carbohydrate to protein present in each sample was calculated by dividing the micrograms of carbohydrate

by the micrograms of protein in the sample. This ratio calculation demonstrated that HBV antigens produced in mnn9⁻ recombinant yeast cells consistently contained one tenth of the carbohydrate content of HBV antigens produced in recombinant "wild-type" yeast cells. These results show that HBV antigens produced in the mnn9⁻ mutant yeast cells contained substantially reduced amounts of carbohydrate when compared to HBV antigens produced in "wild-type" yeast cells.

It may be advantageous to prepare a DNA ORF encoding HBcAg lacking the C-terminal nucleic acid binding region. This DNA ORF will result in the production of a core protein which does not bind nucleic acid, and will form a particle without high levels of nucleic acid. HBcAg deletion mutants which do not bind nucleic acids, and form particles consisting only of HBcAg have been produced by recombinant DNA techniques [Gallina et al., supra; and Birmbaum et al., supra].

In addition, full length HBcAg which retains the nucleic acid binding domain may be treated in such a manner as to degrade or displace the bound nuleic acid. Treatment methods to remove bound nucleic acid from HBcAg include, but are not limited to, chemical degradation of the nucleic acid (e.g. using alkali), enzymatic degradation of the nucleic acid (e.g. deoxyribonucleases, ribonucleases) and displacement of nucleic acid by other non-nucleic acid compounds (e.g. polyanionic compounds).

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1

Cloning of HBV DNA in pBR322

HBV Dane particles (serotype adw and ayw) were isolated and purified from human plasma (carrier), and double-stranded DNA was synthesized by the endogenous polymerase in the Dane particles according to the methods of Landers et al., [J. Virology, 23, 368-376, (1977)] and Hruska et al., [J. Virology, 21, (1977)]. The DNA was isolated after digestion with Proteinase K in SDS followed by extraction with phenol/chloroform and ethanol precipitation. The HBV genomic DNA was digested with EcoRI, producing a single 3.2 kbp fragment, that was cloned into the EcoRI site of pBR322 to form pHBV/ADW-1 or pHBV/AYW-1. The presence of the HBV DNA was confirmed by EcoRI digestion, Southern blot transfer to nitrocellulose, and hybridization with [^{32}P]-labelled specific oligonucleotide probes.

EXAMPLE 2

Cloning of the HBcAg Gene into the pGAP-tADH-2 Expression Vector pC1/1, and pUC vectors

As shown in Figure 1, plasmid HBV/AYW-1 (described in Example 1) was digested with HhaI and AvaI and the 0.6 kbp fragment purified by preparative agarose gel electrophoresis. After subsequent

digestion with TaqI, the resulting 0.5 kbp fragment also was purified by preparative agarose gel electrophoresis to yield a fragment with TaqI and AvaI termini.

To reconstruct the 5' portion of the HBcAg ORF, a pair of oligonucleotides were synthesized which reconstitutes the ORF from the TaqI site upstream to the ATG through a 10bp NTL sequence to a HindIII terminus. The sequence of this oligonucleotide is:

AGCTTACAAAACAAAATGGACAT (SEQ.ID.NO.2)

ATGTTTTGTTTTACCTGTAGC (SEQ.ID.NO.3)

To reconstitute the 3' portion of the HBcAg ORF, a second pair oligonucleotides were synthesized which reconstitutes the ORF from the AvaI site through the translational terminator to a HindIII terminus. The sequence of this oligonucleotide is:

TCGGGAATCTCAATGTTAGA (SEQ.ID.NO.:4)

CTTAGAGTTACAATCTTCGA (SEQ.ID.NO.:5)

The plasmid pGAP-tADH-2 (see Fig. 1) containing the GAP491 promoter [Holland and Holland, J. Biol, Chem. 255: 2596 (1980)] and the ADH1 transcriptional terminator in pBR322, has a unique HindIII cloning site into which the HBcAg ORF described above was ligated, yielding pEGC-1 (Figure 1). The presence and orientation of HBcAg DNA was confirmed by restriction endonuclease analyses and Southern blot hybridization. The expression cassette containing the HBcAg ORF was removed from pEGC-1 by SphI digestion and isolated by preparative agarose gel electrophoresis. The cassette was then cloned into the shuttle vector pC1/1 [Beggs, Nature 275: 104

(1978); Rosenberg et al., Nature, 312: 77 (1984)] which had been digested previously with SphI (pYGC-1).

The resultant pC1/1 plasmid containing the expression cassette was digested with HindIII and the complete HBcAg open reading frame (ORF) was isolated and purified (0.57 kbp) by preparative agarose gel electrophoresis. The HBcAg ORF was ligated into HindIII digested pUC vector; this allowed its subsequent intact removal by digestion with HindIII and cloning into different expression vectors. The HBcAg ORF was ligated into the vector containing the ADH2 promoter (Kniskern et al., 1988 Hepatology 8, 82-87) and the orientation was confirmed by restriction analysis.

This expression cassette containing the ADH2 promoter, the HBcAg ORF and ADH1 terminator was cloned into the shuttle vector pC1/1 (Figure 2; Beggs, supra; Rosenberg, et al., supra) to create a yeast expression vector which was then used to doubly transform S. cerevisiae as described below.

EXAMPLE 3

Cloning of preS1+preS2+S (ayw) ORF into the GAL10 Expression Vector

The preS1+preS2+S cloning vector (as described by Kniskern et al., 1988, Hepatology, 8, pp82-87) was digested with HindIII followed by isolation and purification of the 1.17kb DNA fragment by agarose gel electrophoresis. This DNA was ligated into the HindIII site of the GAL10 promoter expression vector. The GAL10 expression vector was

prepared by the substitution of the 1.1 kbp GAP promoter (Example 2) with the GAL10 promoter (0.5kbp) [Schultz, L.D. et al., 1987, Gene, 54, pp. 113-23]. The ligated vector was used to transform E. coli and transformants were screened for the appropriate plasmid containing the preS1+preS2+S ORF in the correct orientation. The expression cassette (GAL10 promoter, the DNA ORF, and the ADH1 terminator) was digested with SphI and the 2.0kbp DNA fragment was isolated and purified by agarose gel electrophoresis. The cassette was ligated into the SphI site of the shuttle vector pYEp24 (New England Biolabs) to create a yeast expression vector (Figure 3) which was then used to doubly transform S. cerevisiae as described below.

EXAMPLE 4

Glycosylation Mutant (Asn to Gln) in PreS1+PreS2+S ORF

The glycosylation mutation was introduced into the preS1+preS2+S ORF by a pair of oligonucleotides (Gln 4 instead of Asn 4). Asn 4 is the only amino acid that is N-glycosylated in the

yeast host. Changing the Asn to Gln (Q4) results in a mutant that is not N-glycosylated. The preS1+preS2+S cloning vector has been described previously (Kniskern et al., 1988, Hepatology, 8, pp82-87). Two intermediate cloning vectors were used in the assembly of the preS1+preS2+S ORF. An EcoRI site divides the complete preS1+preS2+S coding region into a 5' domain of 0.4kbp and a 3' domain of 0.8kbp. These two domains were subcloned separately for eventual reassembly of the entire gene. The 3' domain was cloned in pUC19/DSD which contains most of preS2 and all of S (EcoRI to HindIII). The 5' domain (pUC18/USD) contains all of preS1 and a few bases of preS2 (HindIII to EcoRI). To introduce the amino acid change, the 5' end of the preS1 ORF (including Asn 4) was replaced by a pair of oligonucleotides (72-mers) which reconstitute the ORF from the BamHI site upstream to the ATG through a 10bp NTL sequence, through the HindIII terminus. The sequence of the oligonucleotides are shown below:

```
AGCTTACAAA ACAAAATGGG GCAGCAGCTT TCCACCAGCA
         10         20         30         40
ATCCTCTGGG ATTTTTTCCC GACCACCAGT TG (SEQIDNO:6)
         50         60         70 72

ATGTTTTGTT TTACCCCGTC GTCGAAAGGT GGTCGTTAGG
         10         20         30         40

AGACCCTAAA AAAGGGCTGG TGGTCAACCT AG (SEQIDNO:7)
         50         60         70 72
```

The pUC18/USD vector was restricted with HindIII and BamHI enzymes and the resultant vector (2.9kb) was isolated and purified by agarose gel electrophoresis. The oligonucleotide pair was annealed and ligated to the digested vector. The resultant vector was digested with HindIII and EcoRI. The preS1 DNA(0.4kbp) was purified by agarose gel electrophoresis.

The 3' domain of the ORF was isolated from pUC19/DSD by digestion with EcoRI and HindIII (0.8kbp). The 5' and 3' fragments were ligated, digested with HindIII and the complete ORF (as a HindIII fragment, 1.2 kbp) was isolated by agarose gel electrophoresis. The ORF was sequenced to verify the mutation. The HindIII fragment was ligated into the GAL10 expression vector as described in Example 3. The expression cassette containing the specific promoter (GAL10), the preS1+preS2+S (Q4) ORF, and the ADH-1 terminator was cloned into the shuttle vector YEp24 (Figure 3).

EXAMPLE 5

Construction of the Inducible Bidirectional Vector Expressing the preS1+preS2+S ORF and the HBcAg

Plasmid pBM272 which contains the GAL1/GAL10 promoter fragment in YCp50 [M. Johnston and R. W. Davis (1984) Mol. Cell. Biol. 4, pp.1440-1448; G. Barnes and J. Rine (1985) PNAS 82, pp.1354-1358] was digested with restriction endonucleases HindIII and SphI and was ligated with a 0.35kbp DNA fragment with HindIII-SphI ends containing the ADH1 terminator.

This ligated plasmid was used to transform E. coli and transformants were screened for the plasmid containing the ADH1 terminator. This plasmid was digested with EcoRI and SalI, and the 1.1kbp DNA fragment containing the GAL1/GAL10 promoter region with the ADH1 terminator downstream from the GAL1 promoter was isolated and purified by preparative agarose gel electrophoresis.

Plasmid pUC18GAL10tADH1 was digested with HindIII and EcoRI to remove the GAL10 promoter region and the large vector fragment was agarose gel purified. Two synthetic DNA oligonucleotides were synthesized which have the following sequence:

AATTGTCGAC AGCTAGCTGA ATTCCCGGG (SEQIDNO: 8)
1 10 20 29

AGCTCCCGGG AATTCAGCTA GCTGTCGAC (SEQIDNO: 9).
1 10 20 29

These two oligonucleotides were annealed and ligated to the HindIII - EcoRI digested pUC18GAL10tADH1 vector. The synthetic oligonucleotide described above, when ligated to the pUC18GAL10tADH1 vector as described, destroys the EcoRI and HindIII sites of the vector, and adds SalI, EcoRI and SmaI recognition sites to the vector.

The vector containing the ligated synthetic oligonculeotide was digested with SalI and EcoRI and the vector was purified by agarose gel electrophoresis. Into this vector was ligated the SalI-EcoRI DNA fragment containing the ADH1 terminator downstream from the GAL1 promoter, plus the GAL10 promoter. This ligated vector was used to transform E. coli and transformants were screened for

the presence of the appropriate plasmid construction. The resulting plasmid contains the GAL1/GAL10 promoter region with a ADH1 terminator downstream for each promoter (see Figure 4). Cloning sites between the GAL10 promoter and the ADH1 terminator are EcoRI and SmaI, and cloning sites between the GAL1 promoter and the ADH1 terminator are BamHI and HindIII.

The preS1(Q4)+preS2+S ORF (Example 4) was obtained from the cloning vector by digestion with HindIII and agarose gel purification of the 1.17kbp DNA fragment. This fragment was blunt end ligated into the SmaI site of the bidirectional vector downstream from the GAL10 promoter. The ligated vector was used to transform E. coli and transformants were screened for the appropriate plasmid containing the preS1+preS2+S ORF in the correct orientation.

The HBcAg ORF was obtained from the pUC-based plasmid (Example 2) by digestion with HindIII and agarose gel purification of the 0.57kbp DNA fragment. This fragment was ligated into the HindIII site of the bidirectional vector (containing the preS1+preS2+S ORF) downstream from the GAL1 promoter.

The ligated vector was used to transform E. coli and transformants were screened for the appropriate plasmid containing both the preS1+preS2+S ORF and the HBcAg ORF in the correct orientations. The expression cassette containing the HBcAg, the preS1+preS2+S ORF, and the pGAL1/pGAL10 promoter region, and the ADH1 terminators was digested with SphI and the 3.17kbp DNA was isolated and purified by agarose gel electrophoresis. The DNA was ligated between the SphI sites of the pC1/1 shuttle vector.

The final plasmid construction is shown in Figure 4.

Example 6

Elimination of the Nucleic Acid Binding Domain from HBcAg

Elimination of the nucleic acid binding domain of the core antigen was achieved by the removal of the C-terminal thirty-four (34) amino acids (Arg-rich region). This was achieved by the removal from the core DNA ORF, the 3' terminal portion that codes for these amino acids, and was done as follows: The core antigen-encoding DNA from Example 2 was digested with EcoRI and AvaI and the 0.55 kbp fragment was purified by preparative agarose gel electrophoresis. Subsequently, this DNA was digested with HpaII and the 0.44kbp DNA fragment was purified by agarose gel electrophoresis. This restriction digestion removes all the DNA encoding the Arg-rich region of core antigen as well as an additional 6 amino acids. To reconstruct the 3' portion of the core ORF, a pair of DNA oligonucleotides were synthesized which reconstituted the ORF from the HpaII site by adding the 6 amino acids, a translational terminator and a HindIII terminus. The sequence of these oligonucleotides are given below:

1. 5'- CGGAG ACT ACT GTT GTT TAG A -3'
   (SEQ.ID.NO.: 10)

2. 5'- AGCTT CTA AAC AAC AGT AGT CTC -3'
   (SEQ.ID.NO.: 11)

The cloning vector (pGEM3) was prepared by digestion with EcoRI and HindIII restriction enzymes. The above oligonucleotides were annealed and ligated into the vector. The vector with the oligonucleotides was purified by agarose gel electrophoresis; the vector now has EcoRI-HpaII ends and can accept the core-encoding DNA (0.44 kbp) which is EcoRI/HpaII ended. This vector contains the abbreviated core ORF with HindIII termini. The DNA can be placed downstream from a promoter (e.g. ADH2 promoter of yeast) and into a yeast shuttle vector.

EXAMPLE 7

Construction of yeast S. Cerevisiae CF52 and CF55 (mnn9=) mutant yeast strains

Yeast S. cerevisiae strain KHY 107 (cir$^+$, ade1$^+$, leu2$^-$ and mnn9$^-$) was constructed as follows: The α mating type strain CZ5/LB347-1C (mnn9$^-$, Suc2) was mated with the "a" type strain 2150-2-3 (leu2$^-$, ade1$^-$) by mixing the strains on a YEHD complete media plate. To select for diploids, the mated strains were replica plated onto leu$^-$ minimal medium containing 2% sucrose as the sole carbon source. After isolating single colonies, the diploids were sporulated, and asci were dissected by standard techniques. The KHY-107 strain was isolated as a single spore and characterized as cir$^+$, ade1$^+$, leu2$^-$, and mnn9$^-$ (by Schiff stain technique).

KHY107 (cir 0) was derived from strain KHY107 (cir$^+$) as described by Broach [Methods in Enzymology, 101, Part C, pp 307-325, (1983)]. The

cured strain was made ura3⁻ by integrating a disrupted ura3 gene. The resulting strain, KHY-107ura3Δ, was grown in rich media to allow the accumulation of spontaneous mutations and a canavanine resistant mutant was selected. The mutant strain, CF55 (Mata leu2-2,112 ura3Δ can1 mnn9⁻ cir°), was shown by complementation tests to be can1⁻. The GAL10pGAL4 expression cassette was integrated into the HIS3 gene of CF55 (Methods in Enzymology, 1990, 185 pp297-309) to yield the final host strain CF52 (Mata leu2-2,112 ura3Δ can1 his3Δ::GAL10pGAL4-URA3, cir° mnn9⁻).

EXAMPLE 8

Yeast Transformation and Seed Establishment for Mixed Particles of HBcAg and preS1+preS2+S in CF55 mnn9⁻ Mutant Yeast

The pC1/1pADH2-HBcAg-tADH-1 plasmid described in Example 2 and the YEp24pGAL10-preS1+preS2+S-tADH-1 plasmid described in Example 4 were used together to double transform S. cerevisiae strain CF55 (Example 7). Clones were selected on minimal medium (leu-, ura-, containing 1M sorbitol), established as frozen stocks (in 17% glycerol) and evaluated as described below.

EXAMPLE 9

Yeast Transformation and Seed Establishment for HBcAg in CF52 mnn9⁻ Mutant Yeast

The bidirectional vector described in Example 5, was used to transform S. cerevisiae strain

CF52. Clones were selected on minimal medium (leu- containing 1M sorbitol), established as frozen stocks (in 17% glycerol) and evaluated as described below.

EXAMPLE 10

Growth and Expression of Mixed Particles in Yeast CF55 and CF52 (mnn9$^-$)

Clones of yeast containing the expression plasmid described in Examples 2, 4, and 5, above were plated onto leu$^-$ selective agar plates containing 1M sorbitol and incubated at 30°C for 2-3 days. These yeast were inoculated with 5-7 mL of complex YEHDS (YEHD + 0.1M sorbitol) and the cultures were incubated at 30°C with aeration for 12-18 hours. Flasks containing 50 mL YEHDS + 2% galactose media were inoculated from the above culture (to an initial A_{600} = 0.1) and incubated at 30°C with shaking (350 rpm) for 72 hours to a final A_{600} of 10-16. Samples of 10 A_{600} units were dispensed into tubes, and the yeast cells were pelleted at 2,000 x g for 10 minutes. The pellets either were assayed directly or stored at -70°C for future assay. At the time of assay, the pellets were resuspended in 0.3 mL of phosphate-buffered saline containing 2mM PMSF. Yeast cells were broken by: 1) the addition of 200-300 mg of washed glass beads (0.45 mm), 2) agitation on a vortex mixer for 15 minutes, 3) addition of TRITON X-100 to 0.5% (v/v), 4) agitation on a vortex mixer for 2 minutes, and 5) incubation at 4°C for 10-15 minutes. Cellular debris and glass beads were removed by centrifugation at 13,000 x g for 10

minutes. The clarified supernatant fluid was removed and analyzed for protein [by the method of Lowry et al., J. Biol. Chem., 193, 265 (1951)] and for HBsAg by (AUSRIA^R) assay (Abbott) and for preS2+S by specific RIA assay (Hansson, et al., supra; Machida, et al., supra), and for HBcAg by CorAg^R (Abbott). The results are shown in Table 1, and demonstrate the presence of HBcAg and preS1+preS2+S(Q4) in the particles.

Clarified lysates also contained 28 nm particles, typical of HBcAg, which were shown to display pre-S determinants based on immunoelectron microscopy (micrographs shown in Figure 5). Typical assay results are shown below.

TABLE 1

DOUBLE EXPRESSERS

preS1+preS2+S with HBcAg

CONSTRUCT	RATIO[1]	CORAG	AUSRIA	S+preS	C+preS
Two vectors	1:10	++	+	+	+
BDV	1:1	++	+	+	++
HBcAg	N.A.	+++	-	-	-

[1]preS1+preS2+S: C ; based on expected plasmid copy numbers and relative promoter strengths.

EXAMPLE 11

Large Scale Growth of S. cerevisiae Producing HBV mixed particles in Fermentors

The frozen recombinant yeast culture was inoculated onto leu$^-$ plates containing 1M sorbitol. The plates were incubated inverted at 28°C for 2-3 days. The growth on the plates was resuspended in YEHDS and the resuspended growth was transferred into 2-L Erlenmeyer flask containing 500 mL of YEHDS, and 2% galactose. The flask was incubated at 28°C and 350 rpm in a controlled environment shaker incubator for 18-22 hours. These seed cultures then were used to inoculate the production stage vessels.

An inoculum (1-5% v/v) from one or more flasks was transferred into 16-L or 250-L fermentors containing 10-L or 200-L of YEHDS, respectively. The 16-L fermentors were operated at 500 rpm, 5 L/min air, and 28°C. The 250-L fermentors were operated at 160 RPM, 60 L/min air and 28°C. The fermentors were harvested 40-46 hrs after inoculation with the seed culture. Optical density values of 15.0 A_{660} units typically were obtained. Harvesting consisted of concentrating the cells using a hollow fiber filtering device followed by washing the cells in buffered salt solutions. Cell slurries were assayed as described below or stored frozen at -70°C for further processing and analysis.

Small samples (0.6 mL) of 20% washed cell slurries were broken using glass beads (0.45-0.52 mm) in 1.5-mL Eppendorf tubes. PMSF (6.5 µl of 200 mM stock) was added as a protease inhibitor. Aliquots were removed from the tubes after breakage and frozen

at -70°C for immunoblot analysis. TRITON X-100 was added to the remaining sample in the tubes to a final concentration of 0.5%, and the samples were mixed briefly and incubated at 4°C for 20-40 min. The cell debris was removed by centrifugation and the clarified cell extract assayed for S antigen by (Ausria^R), preS by RIA, and protein (Lowry) and C by CorAg^R.

EXAMPLE 12

Purification of Mixed particles in particulate form by means of immune affinity chromatography

Recombinant S. cerevisiae, constructed as described in Examples 8 and 9, were grown in either flasks or fermentors. The yeast cells were harvested by microfiltration in an Amicon DC-10 unit, suspended in 30 ml buffer A [0.1M Na_2HPO_4, pH 7.2, 0.5M NaCl], and broken in a Stansted pressure cell for seven passages at 75-85 pounds per square inch. The broken cell suspension (31 gm wet cell weight) was diluted with 120 ml buffer A, Triton X-100 was added to a final concentration of 0.5% (w/v), and the suspension was clarified by centrifugation at 10,000 x g for 20 min. at 4°C. The clarified broth was decanted and incubated with Sepharose 4B coupled with antibodies to HBsAg [McAleer et al., Nature 307: 178 (1984)] for 19 hours at 4°C to adsorb the antigen onto the resin. After the incubation period, the slurry was warmed to room temperature for all subsequent steps and degassed under vacuum for 15 min. The degassed slurry was poured into a 2.5 x 40 cm column. When the column had been packed fully, unbound protein was

washed away with buffer A. The antigen was eluted with 3M KSCN in buffer A. Fractions containing antigen were dialyzed against 0.007M Na_2HPO_4, pH 7.2, 0.15M NaCl at 4°C and pooled to form the Dialyzed Affinity Pool containing 1.08 mg of protein in 20 ml. Sixteen ml of Dialyzed Affinity Pool was diluted to 40 mcg/ml with 5.6 ml 0.006M Na_2HPO_4, pH 7.2, 0.15M NaCl. The product was sterilized by filtration through a Millex-GV 0.22 μ membrane. The identity of the product in the Dialyzed Affinity Pool was verified by the detection of HBsAg by Ausria^R reactivity, and by the detection of preS by RIA and C by CorAg^R.

EXAMPLE 13

Removal of Nucleic Acid From HBcAg-containing Particles By Base Digestion

Nucleic acids can be removed from HBcAg by use of nucleic acid digestion agents. One such method used digestion with base (NaOH) at .05M (range .005-.5). The purified core particles were treated with base for 1 hour at different temperatures (65°C and 37°C). Nucleic acid could not be detected following base digestion. The removal of nucleic acid was followed by ethidium bromide staining following agarose gel electrophoresis as well as by dot blotting. In the case of dot blotting, core DNA labeled with ^{32}P was used as the probe. The base treatments eliminate core antigen activity and core particles cannot be seen by electron microscopy. It was possible to regain a portion of the core particles and core antigen activity (See Table 2) by addition of a polyanion to replace the nucleic acid. The polyanion used was the H. influenzae, polyribitol phosphate (PRP).

TABLE 2

REMOVAL OF NUCLEIC ACID FROM HBcAg

TREATMENT	RNA	CORAG	28nm PARTICLES
None	ca.10%	+++	+++
Base, then Neutralize	<0.01%	-	-
Base, then PRP, then Neutralize	<0.01%	+	+

EXAMPLE 14

Immuno-electron microscopy

About 25µl of the mixed particles from Example 10 were mixed with 25µl of a 1:30 dilution (in PBS) of monoclonal antibody 10E7-2, specific for preS2, and incubated at 37°C for about 60 minutes. A solution of 2% phosphotungstic acid was added to the mixed particles for negative staining. The electron micrographs are shown in Figure 5(a) and (b), and demonstrate the presence of 28nm particles (5a) containing preS sequences (5b). A Philips 300TEM (operated at 80kv) electron microscope was used with a magnification of 130,000 x.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kniskern, P. J.
Hagopian, A.
Burke, P.

(ii) TITLE OF INVENTION: Vaccine Comprising A Mixed HBV Particle Of preS1+preS2+S And Core Proteins

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Merck & Co., Inc.
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(C) CITY: Rahway
(D) STATE: New Jersey
(E) COUNTRY: US
(F) ZIP: 07065-0900

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Wallen, John W III
(B) REGISTRATION NUMBER: 35,403
(C) REFERENCE/DOCKET NUMBER: 18349

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (908) 594-3905
(C) TELEX: (908) 594-4720

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACAAAACAAA 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCTTACAAA ACAAAATGGA CAT 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGTTTTGTT TTACCTGTAG C 21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCGGGAATCT CAATGTTAGA 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTTAGAGTTA CAATCTTCGA 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCTTACAAA ACAAAATGGG GCAGCAGCTT TCCACCAGCA ATCCTCTGGG ATTTTTTCCC 60

GACCACCAGT TG 72

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGTTTTGTT TTACCCCGTC GTCGAAAGGT GGTCGTTAGG AGACCCTAAA AAAGGGCTGG 60

TGGTCAACCT AG 72

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATTGTCGAC AGCTAGCTGA ATTCCCGGG 29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGCTCCCGGG AATTCAGCTA GCTGTCGAC 29

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGGAGACTAC TGTTGTTTAG A 21

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTTCTAAA CAACAGTAGT CTC 23

WHAT IS CLAIMED IS:

1. Multiple hepatitis B or related virus proteins which form mixed particles containing core protein and envelope proteins and display at least one immunologic epitope of each protein, free from other HBV proteins.

2. The core-containing mixed particles of Claim 1 wherein the particles display the protective "a" epitope of the S protein.

3. The core-containing mixed particles of Claim 2 which contain one or more HBV envelope proteins from HBV serotypes adw, ayw, adr, ayr, adyw or immunologically related variants, selected from the group of envelope proteins consisting of:
the preS1+preS2+S protein;
the preS1(Q4)preS2+S protein;
the preS2+S protein;
the preS2(Q4)+S protein; or
the S protein.

4. The core-containing mixed particles of Claim 3 wherein the ratio of C protein to envelope protein is between approximately 1:20 and 20:1, respectively

5. The core-containing mixed particles of Claim 4 wherein the ratio of C proteins to envelope protein is approximately 1:1, 3:1, 10:1 or 20:1.

6. The core-containing mixed particles of Claim 3 wherein the proteins are simultaneously produced in a recombinant host cell.

7. The core-containing mixed particles of Claim 6 wherein the recombinant host is yeast.

8. The core containing mixed particles of Claim 7 wherein the yeast host is genetically deficient for N-linked protein glycosylation.

9. The core-containing mixed particles of Claim 8 wherein the genetic deficiency of the yeast host is in the MNN9 gene.

10. The core-containing mixed particles of Claim 3 wherein the carbohydrate-to-protein ratio of the purified particles is less than 0.5.

11. The core-containing mixed particles of Claim 10 wherein the envelope protein preS1+preS2+S or preS2+S is non-N-glycosylated.

12. The core-containing mixed particles of Claim 1 wherein the amino acid sequence of the envelope protein is altered to prevent glycosylation.

13. The core-containing mixed particles of Claim 12, wherein the amino acid asparagine of one or more of the N-linked glycosylation sites of the preS1+preS2+S or preS2+S protein is any amino acid other than asparagine.

14. The core-containing mixed particles of Claim 13 wherein the amino acid alteration of the N-linked glycosylation site of the preS1 domain is a substitution of glutamine for asparagine at position 4 of the preS1 amino acid sequence.

15. The core-containing mixed particles of Claim 13 wherein the amino acid alteration of the N-linked glycosylation site of the preS2 domain is a substitution of glutamine for asparagine at position 4 of the preS2 domain amino acid sequence.

16. The core-containing mixed particles of Claim 3 wherein the envelope protein contains an amino acid substitution of glutamine for asparagine at position 4 of the preS1 amino acid sequence, and wherein the ratio of C protein to envelope protein is between approximately 1:20 and 20:1, respectively.

17. The core-containing mixed particles of Claim 3 wherein the envelope protein contains an amino acid substitution of glutamine for asparagine at position 4 of one or both of the pre S1 amino acid sequence and the preS2 amino acid sequence, and wherein the ratio of C protein to envelope protein is between approximately 1:20 and 20:1, respectively.

18. The core-containing mixed particles of Claim 17 wherein the ratio of C protein to preS1+preS2+S protein is approximately 1:1, 3:1, 10:1, or 20:1 respectively.

19. The core-containing mixed particles of Claim 17 wherein the proteins are simultaneously produced in a recombinant host cell.

20. The core-containing mixed particles of Claim 19 wherein the recombinant host is yeast.

21. The core-containing mixed particles of Claim 20 wherein the yeast host is genetically deficient for protein glycosylation.

22. The core-containing mixed particles of Claim 21 wherein the yeast host is genetically deficient for N-linked protein glycosylation.

23. The core-containing mixed particles of Claim 21 wherein the genetic deficiency of the yeast cells is in the MNN9 gene.

24. The core-containing mixed particles of Claim 17 wherein the carbohydrate to protein ratio of the purified particles is less than 0.5.

25. A vaccine comprising multiple hepatitis B virus surface protein and core protein-containing particles according to Claim 1.

26. A vaccine comprising mulitple hepatitis B virus surface protein and core protein-containing particles according to Claim 11.

27. An antigen or immunogen according to Claim 1 for use in development of diagnostic reagents.

28. An antigen or immunogen according to Claim 11 for use in development of diagnostic reagents.

29. An antigen or immunogen according to Claim 24 for use in development of diagnostic reagents.

30. Hepatitis B virus core-containing mixed particles of Claim 1, free from other hepatitis B virus proteins and free from nucleic acids.

31. The hepatitis B virus core protein particles according to Claim 30, wherein nucleic acid levels are less than approximately 10 ng/μg of core protein.

32. The hepatitis B virus core protein particles according to Claim 31, wherein inclusion of nucleic acid within the particle is prevented by elimination of the nucleic acid binding domain of the core protein.

33. The hepatitis B virus core protein particles according to Claim 32, wherein inclusion of nucleic acid is prevented by deleting the C-terminal 34 amino acids of the core protein.

34. The hepatitis B virus core protein particles according to Claim 31, wherein the nucleic acid is removed by digestion of the nucleic acid with a nucleic acid digesting agent.

35. The hepatitis B virus core protein particles according to Claim 34 wherein the digesting agent is a base.

36. The hepatitis B virus core protein particles according to Claim 35 wherein the base is sodium hydroxide at a concentration of between approximately 0.005M and 0.5M.

37. The hepatitis B virus core protein particles according to Claim 36, wherein the concentration of sodium hydroxide is 0.05M.

38. The hepatitis B virus core protein particles according to Claim 34, wherein the nucleic acid digesting agent is an enzyme.

39. The hepatitis B virus core protein particles according to Claim 38, wherein the enzyme is ribonuclease.

40. The hepatitis B virus core protein particles according to Claim 30, wherein the core protein-containing particle incorporates a polyanion to replace nucleic acids.

41. The hepatitis B virus core protein particles according to Claim 40, wherein the polyanion is a carbohydrate.

42. The hepatitis B virus core protein particles according to Claim 41, wherein he carbohydrate is polyriboribitol phosphate.

43. Hepatitis B virus mixed particles containing core protein and envelope proteins displaying at least one immunologic epitope of each protein, free from other HBV proteins and free from nucleic acids.

44. The core-containing mixed particles of Claim 43 wherein the particles display the protective "a" epitope of the envelope protein and contain less than approximately 10 ng nucleic acid/μg protein.

45. The core-containing mixed particles of Claim 44 which contain one or more HBV envelope proteins from HBV serotypes adw, ayw, adr, ayr, adyw or immunologically related variants, selected from the group of proteins consisting of:
the preS1+preS2+S protein;
the preS1(Q4)preS2+S protein;
the preS2+S protein;
the preS2(Q4)+S protein; or
the S protein.

46. The core-containing mixed particles of Claim 45 wherein the ratio of C protein to envelope protein is between approximately 1:20 and 20:1, respectively.

47. The core-containing mixed particles of Claim 46 wherein the ratio of C proteins to envelope protein is approximately 1:1, 3:1, 10:1 or 20:1.

48. The hepatitis B virus core protein particles according to Claim 44, wherein inclusion of nucleic acid within the particle is prevented by elimination of the nucleic acid binding domain of the core protein.

49. The hepatitis B virus core protein particles according to Claim 48, wherein inclusion of nucleic acid is prevented by deleting the C-terminal 34 amino acids of the core protein.

50. The hepatitis B virus core protein particles according to Claim 43, wherein the nucleic acid is removed by digestion of the nucleic acid with a nucleic acid digesting agent.

51. The hepatitis B virus core protein particles according to Claim 50 wherein the digesting agent is a base.

52. The hepatitis B virus core protein particles according to Claim 51 wherein the base is sodium hydroxide at a concentration of between approximately 0.005M and 0.5M.

53. The hepatitis B virus protein particles according to Claim 52, wherein the concentration of sodium hydroxide is 0.05M.

54. The hepatitis B virus core protein particles according to Claim 50, wherein the nucleic acid digesting agent is an enzyme.

55. The hepatitis B virus core protein particles according to Claim 54, wherein the enzyme is ribonuclease.

56. The hepatitis B virus core protein particles according to Claim 43, wherein the core protein particle incorporates a polyanion to replace nucleic acids.

57. The hepatitis B virus core protein particles according to Claim 56, wherein the polyanion is a carbohydrate.

58. The hepatitis B virus core protein particles according to Claim 57, wherein the carbohydrate is polyriboribitol phosphate.

59. The core-containing mixed particles of Claim 45 wherein the proteins are simultaneously produced in a recombinant host cell.

60. The core-containing mixed particles of Claim 59 wherein the recombinant host is yeast.

61. The core containing mixed particles of Claim 60 wherein the yeast host is genetically deficient for protein glycosylation.

62. The core containing mixed particles of Claim 61 wherein the yeast host is genetically deficient for N-linked protein glycosylation.

63. The core-containing mixed particles of Claim 62 wherein the genetic deficiency of the yeast host is in the mnn9 gene.

64. The core-containing mixed particles of Claim 45 wherein the carbohydrate-to-protein ratio of the purified particles is less than 0.5.

65. The core-containing mixed particles of Claim 45 wherein the envelope protein preS1+preS2+S or preS2+S is non-N-glycosylated.

66. The core-containing mixed particles of Claim 65 wherein the amino acid sequence of the envelope protein is altered to prevent N-glycosylation.

67. The core-containing mixed particles of Claim 66 wherein the amino acid alteration of the envelope protein preS1 domain is a substitution of glutamine for asparagine at position 4 of the preS1 amino acid sequence.

68. The core-containing mixed particles of Claim 67 wherein the amino acid alteration of the envelope protein preS2 domain is a substitution of glutamine for asparagine at position 4 of the preS2 amino acid sequence.

69. The core-containing mixed particles of Claim 45 wherein the preS1+preS2+S protein contains an amino acid substitution of glutamine for asparagine at position 4 of the preS1 amino acid

sequence, and wherein the ratio of C protein to preS1+preS2+S protein is between approximately 1:20 and 20:1, respectively.

70. The core-containing mixed particles of Claim 45 wherein the preS2+S protein contains an amino acid substitution of glutamine for asparagine at position 4 of the preS2 amino acid sequence, and wherein the ratio of C protein to preS2+S protein is between approximately 1:20 and 20:1, respectively.

71. The core-containing mixed particles of Claim 70 wherein the ratio of C protein to preS1+preS2+S protein is approximately 1:1, 3:1, 10:1, or 20:1 respectively.

72. The core-containing mixed particles of Claim 70 wherein the proteins are simultaneously produced in a recombinant host cell.

73. The core-containing mixed particles of Claim 72 wherein the recombinant host is yeast.

74. The core-containing mixed particles of Claim 73 wherein the yeast host is genetically deficient for protein glycosylation.

75. The core-containing mixed particles of Claim 74 wherein the yeast host is genetically deficient for N-linked protein glycosylation.

76. The core-containing mixed particles of Claim 75 wherein the genetic deficiency of the yeast cells is in the mnn9 gene.

77. The core-containing mixed particles of Claim 45 wherein the carbohydrate to protein ratio of the purified particles is less than 0.5.

78. A vaccine comprising multiple hepatitis B virus surface protein and core protein-containing particles according to Claim 3.

79. A vaccine comprising mulitple hepatitis B virus surface protein and core protein-containing particles according to Claim 30.

80. A vaccine comprising mulitple hepatitis B virus surface protein and core protein-containing particles according to Claim 45.

81. An antigen or immunogen according to Claim 3 for use in development of diagnostic reagents.

82. An antigen or immunogen according to Claim 30 for use in development of diagnostic reagents.

83. An antigen or immunogen according to Claim 45 for use in development of diagnostic reagents.

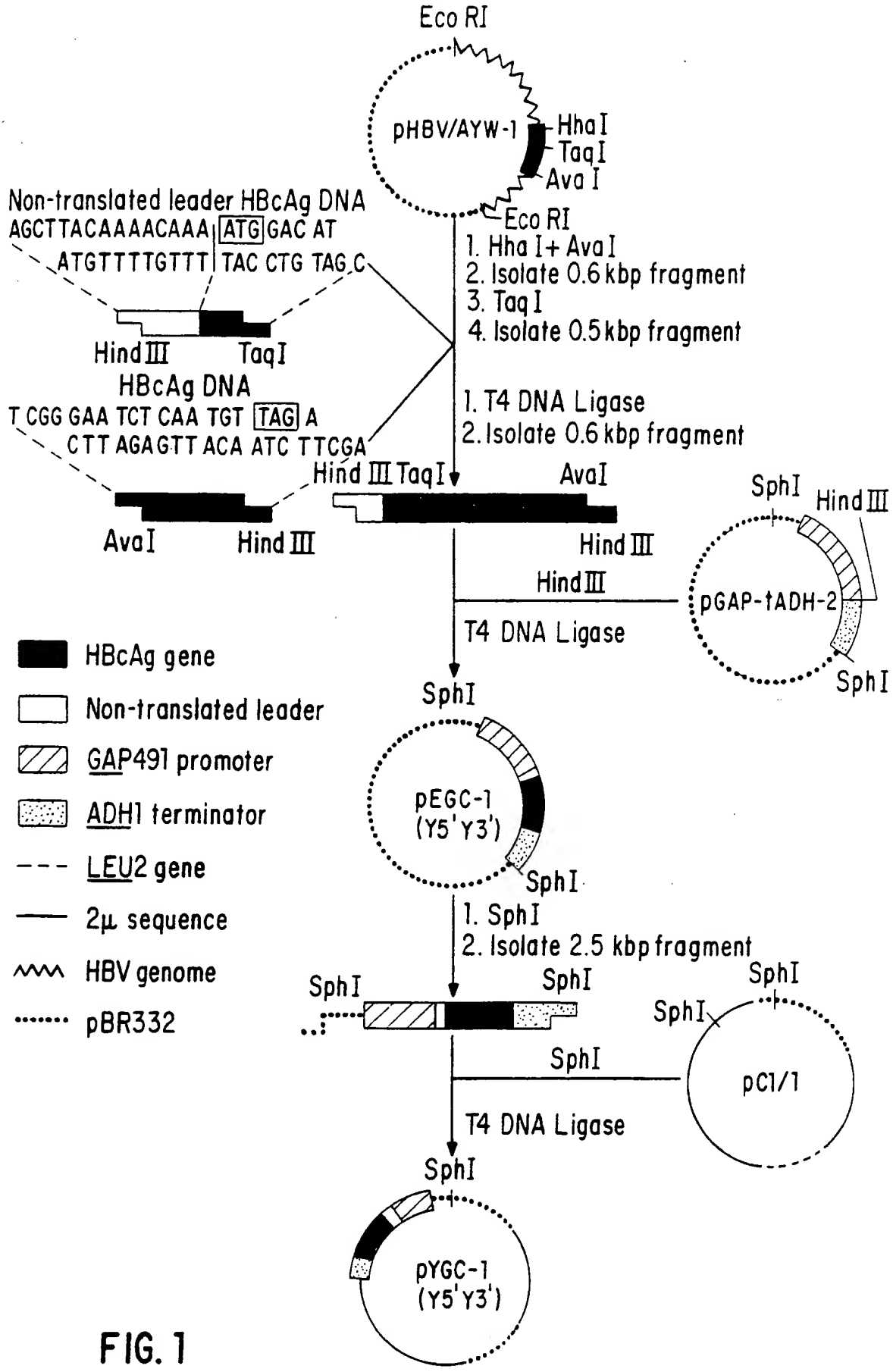
Eco RI
pHBV/AYW-1
Hha I
Taq I
Ava I
Eco RI
Non-translated leader HBcAg DNA
AGCTTACAAAACAAA ATG GAC AT
ATGTTTTGTTT TAC CTG TAG C
Hind III
Taq I
HBcAg DNA
T CGG GAA TCT CAA TGT TAG A
CTT AGAGTT ACA ATC TTCGA
1. Hha I + Ava I
2. Isolate 0.6 kbp fragment
3. Taq I
4. Isolate 0.5 kbp fragment
1. T4 DNA Ligase
2. Isolate 0.6 kbp fragment
Hind III Taq I
Ava I
Ava I
Hind III
Hind III
Hind III
pGAP-tADH-2
Sph I
Hind III
Sph I
T4 DNA Ligase
HBcAg gene
Non-translated leader
GAP491 promoter
ADH1 terminator
LEU2 gene
2μ sequence
HBV genome
pBR332
Sph I
pEGC-1
(Y5'Y3')
Sph I
1. Sph I
2. Isolate 2.5 kbp fragment
Sph I
Sph I
Sph I
Sph I
pC1/1
Sph I
T4 DNA Ligase
Sph I
pYGC-1
(Y5'Y3')

FIG. 1

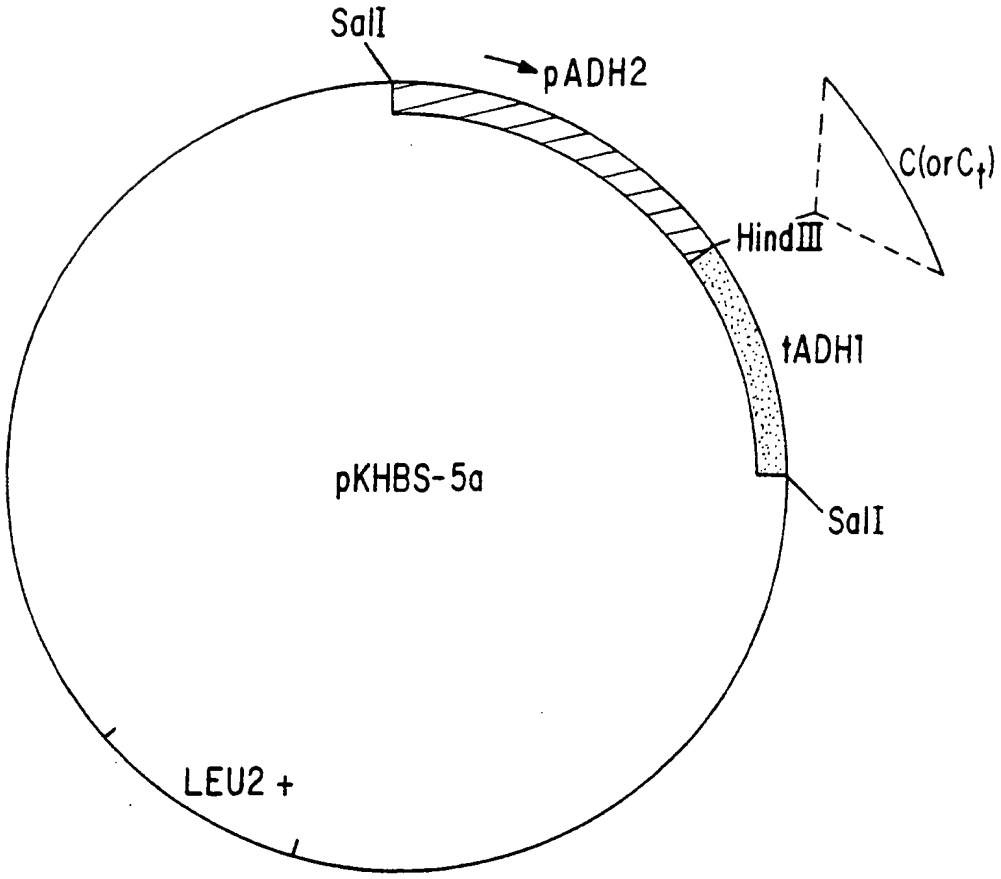

FIG. 2

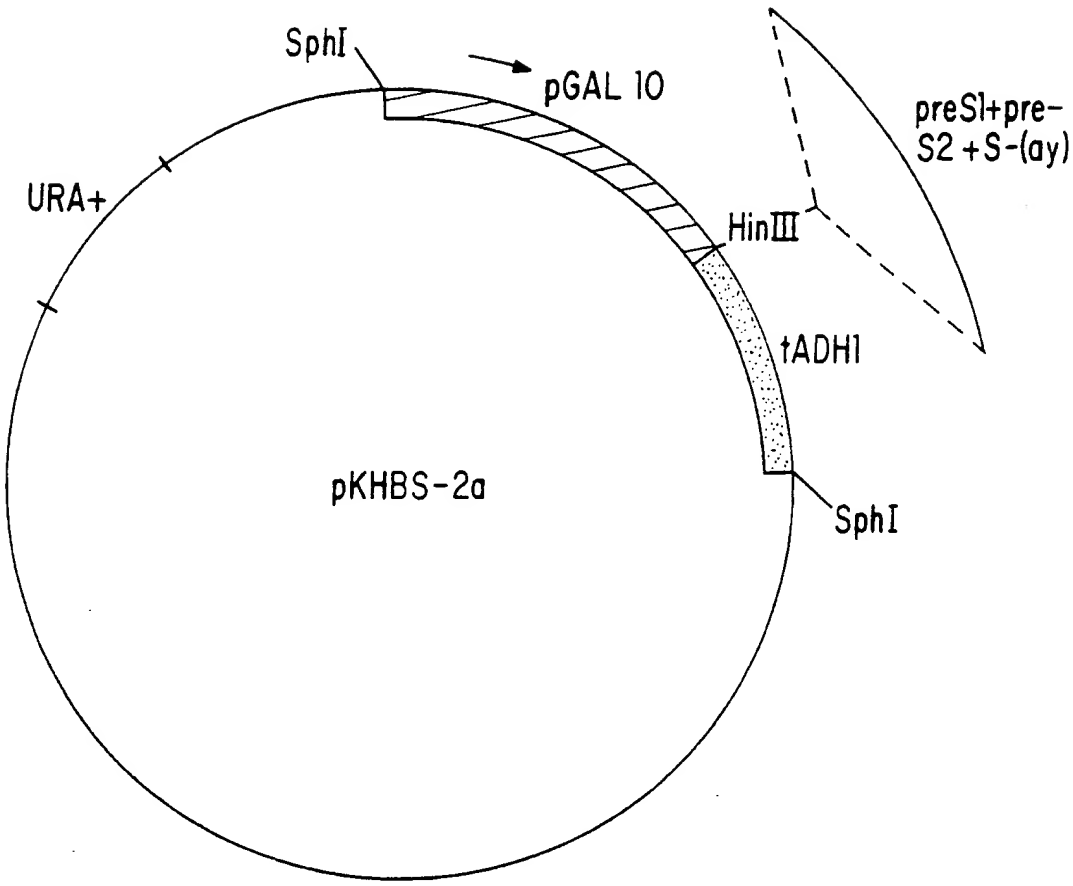

FIG. 3

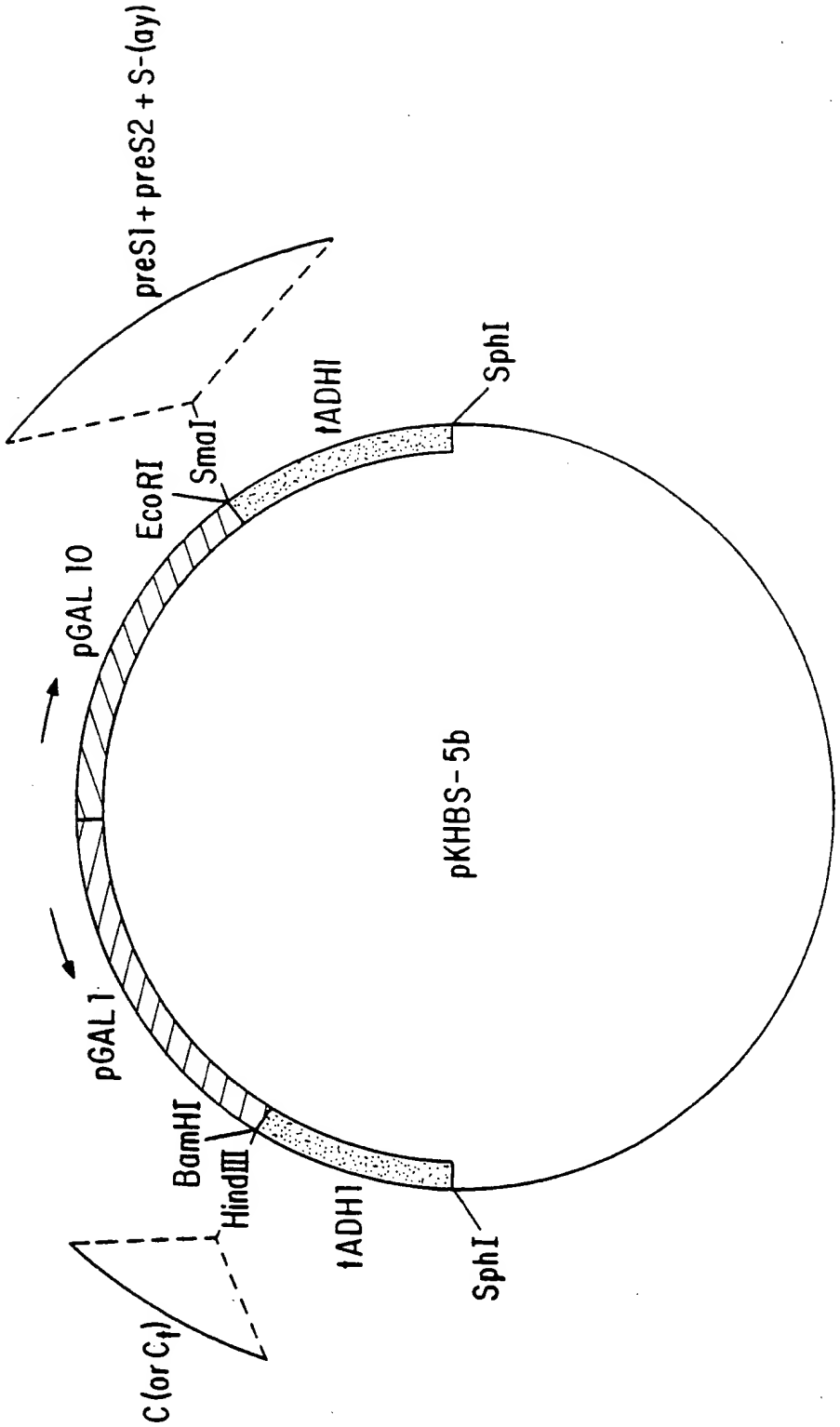
preS1+preS2+S-(ay)
SphI
tADHI
SmaI
EcoRI
pGAL 10
pKHBS-5b
pGAL 1
BamHI
HindIII
tADH1
SphI
C(or Cf)

FIG. 4

FIG.5a

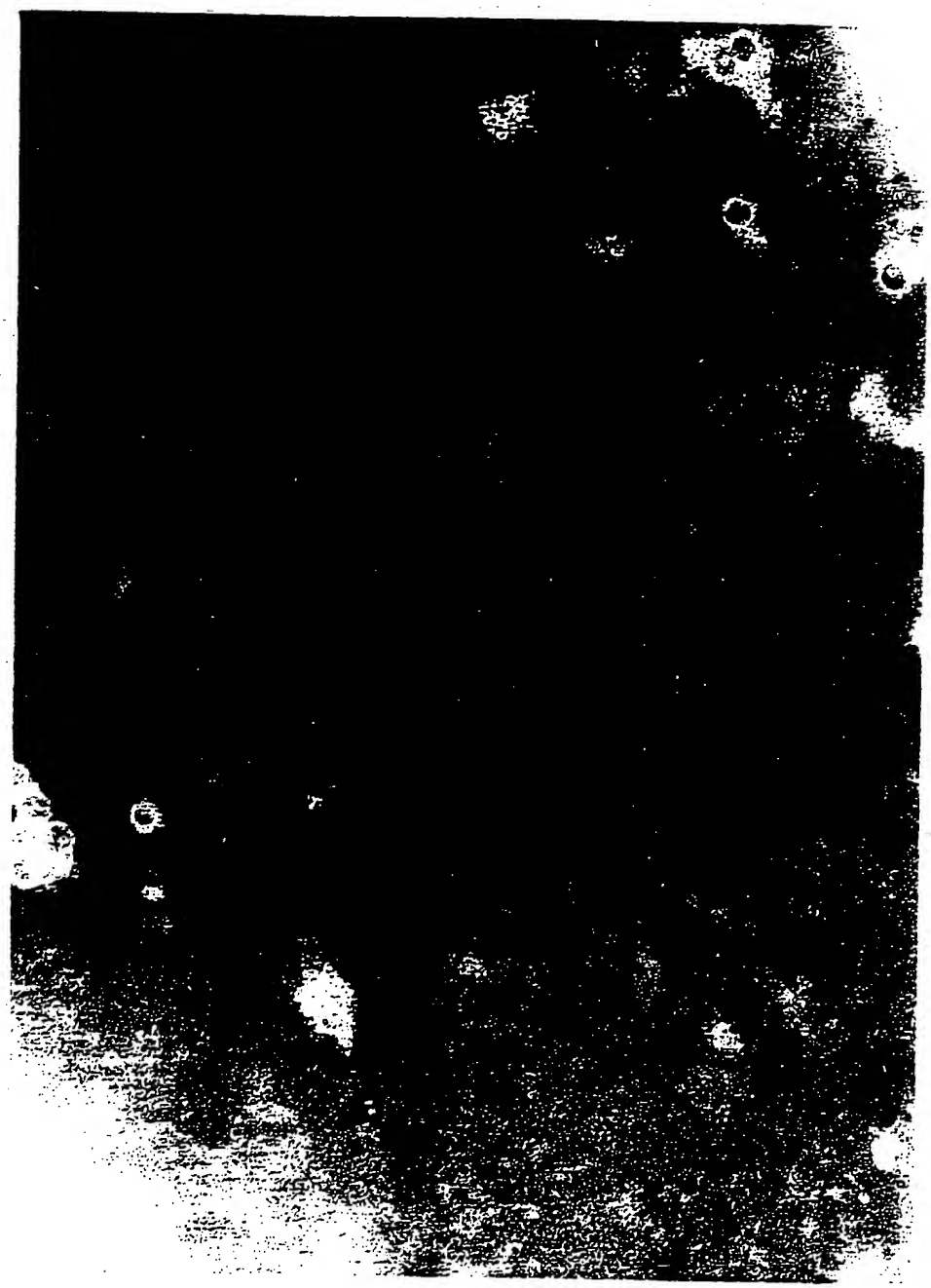

FIG.5b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06252

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/29; C07K 15/04, 13/00, 15/14; C12N 15/00, 15/33, 15/51
US CL :424/88, 89; 530/350, 395, 403, 806, 826, 858; 435/236; 436/820
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 89; 530/350, 395, 403, 806, 826, 858; 435/236; 436/820

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, DERWENT
search terms:surface antigen, core, particle, HBV

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Gene, Volume 106, issued October 1991, Shiosake et al, "Production of Hepatitis B Virion-Like Particles in Yeast", pages 143-149, especially pages 146 and 148.	2-7,10, 25,27,78,81 3,8,9,11-24,26,28-29,34-42,45-47,50-53,56-77,80-81
Y	Gene, Volume 46(1), issued 1986, Kniskern et al, "Unusually High-Level Expression of a Foreign Gene (Hepatitis B Virus Core Antigen) in *Saccharomyces cerevisiae*", pages 135-141, see entire document.	1

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
18 AUGUST 1993

Date of mailing of the international search report
29 SEP 1993

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Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Hepatology, Volume 8(1), issued 1988, Kniskern et al, "A Candidate Vaccine for Hepatitis B Containing the Complete Viral Surface Protein", pages 82-87, see entire document.	1
Y	Postgraduate Medical Journal, Volume 63(2), issued 1987, Petre et al, "Development of a Hepatitis-B Vaccine from Transformed Yeast Cells", pages 73-81, see entire document, especially page 76.	30-77,79,80,82-83
Y	Nature, Volume 282, issued December 1979, Pasek et al, "Hepatitis B Virus Genes and Their Expression in *E. coli*", pages 575-579, see entire document, especially page 577.	30-77,79,80,82,83
Y	Nature, Volume 329, issued October 1987, Millich et al, "Antibody Production to the Nucleocapsid and Envelope of the Hepatitis B Virus Primed be a Single Synthetic T Cell Site", pages 547-549, see entire document, especially page 549.	30-77,79,80,82,83
Y	Journal of Virology, Volume 64(7), issued July 1990, Birnbaum et al, "Hepatitis B Virus Nucleocapsid Assembly: Primary Structure Requirements in the Core Protein", pages 3319-3330, see entire document, especially page 3327.	30-77,79,80,82,83
Y	Journal of Virology, Volume 63(1), issued November 1989, Gallina et al, "A Recombinant Hepatitis B Core Antigen Polypeptide with the Protamine-Like Domain Deleted self-Assembles into Capsid Particles but fails to Bind Nucleic Acids", pages 4645-4652, see entire document, especially Figure 5.	30-77,79,80,82,83
Y	EP, A, 0,314,096 (MacKay et al) 03 May 1989, see entire document, especially page 1198.	3,8,9,11-24,26,28-29,45-47,59-77,80-81
Y	Biochimica et Biophysica Acta, Volume 799, issued June 1984, Lehle et al, "Primary Structural requirements for *N*- and *O*-Glycosylation of Yeast Mannoproteins", pages 246-251, see entire document.	3,11-24,26,28-29,45-47,59-77,80-81
Y	Gene, Volume 67, issued July 1988, Langley et al, "Characterization of Purified Hepatitis B Surface Antigen Containing Pre-S(2) Epitopes Expressed in *Saccharomyces cerevisiae*", pages 229-245, see entire document.	3,11-24,26,28-29,45-47,59-77,80-81
Y	Science, Volume 245, issued 14 July 1989, Chen et al, "Protein-RNA Interactions in an Icosahedral Virus at 3.0 A Resolution", pages 154-159, see entire document, especially p.157.	40-42,56-58

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06252

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	T. Maniatis et al, "Molecular Cloning", published 1982, Cold Spring Harbor Laboratory, (Cold Spring Harbor, New York, United Sates of America), pages 141-143.	34,38-39,50,54-55
Y	The Lancet, Volume 336, issued August 1990, Carman et al, "Vaccine-Induced escape Mutant of Hepatitis B Virus", pages 325-329, see entire document, especially page 328.	3,45

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/GB 98/02852

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/36 C12N15/62 C12N15/70 C12N1/21 C07K16/08
A61K38/16 A61K39/29 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 389 983 A (ABBOTT LAB) 3 October 1990 see the whole document ---	10,11
Y	WO 94 03615 A (MEDEVA HOLDINGS BV ;KHAN MOHAMMED ANJAM (GB); HORMAECHE CARLOS EST) 17 February 1994 see esp p. 3-16; examples ---	1-11
Y	SHI C -H ET AL: "Gene fusion of cholera toxin B subunit and HBV PreS2 epitope and the antigenicity of fusion protein" VACCINE, vol. 13, no. 10, July 1995, page 933-937 XP004057512 see the whole document --- -/--	1-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 January 1999	04/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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